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HISTOPATHOLOGICAL CHARACTERISTICS AND IMMUNOHISTOCHEMICAL BIOMARKERS OF INVASIVE BREAST CANCER IN ETHIOPIAN WOMEN

A Dissertation Presented in Fulfillment of the Requirement for the Degree of Doctor of Philosophy in Medical Biochemistry

By

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Abstract

Background: Breast cancer has been shown as commonest type of cancer in Ethiopian women. It is a heterogeneous disease in terms of clinical, histopathological and biological characteristics. Some of the molecular differences underlying this heterogeneity were previously discovered by genetic expression profiles and validated by immunohistochemical surrogates. Hence, this study was carried out to analyze breast cancer by histopathological features and immunohistochemical markers, and interrelationship among them in Ethiopian female breast cancer cases.

Method: This study was a retrospective analytical study done on clinically and pathologically confirmed breast cancer cases that underwent modified radical mastectomy (MRM) from June, 2014 – June, 2015. The primary breast tumor tissues with the corresponding axillary lymph nodes were examined for histopathological features including histologic type, tumor size, nodal status and grade. Evaluation of the molecular markers including ER, PR, EGFR and VEGF-A were done by immunohistochemistry using formalin-fixed and paraffin-embeded tissues. Data was analyzed using SPSS 24.0. The p value <0.05 was set to be significant for all statistical tests.

Result: The age range at diagnosis was 22-75 years (mean, 42.14±11.96) and more than 70% of the cases were below 50 years of age. The commonest histologic type was invasive ductal carcinoma (NOS, 89.2%). The frequency of pathologic tumor size was 11.3% for T1, 27.5% for T2, 32.5% for T3 and 28.8% for T4. Majority (74%) of them were lymph node positive. Half of the tumors were Grade III followed by grade II (38%). The IHC positivity for ER, PR, and EGFR were 73.8%, 60.2%, and 21.69% respectively. VEGF-A expression was negative, low, moderate and high in 13.41%, 63.41%, 20.73% and 2.44% of the tumors respectively. Significant associations were observed between age and tumor size (p=0.02), ER and EGFR (*P* = 0.01), PR and EGFR (*P* = 0.04), ER and PR (p< 0.001). No significant correlations were observed between age and histopathologic features (except tumor size), age and molecular markers, the molecular markers and histopathological features (except for PR), VEGF and standard molecular markers, EGFR and PR.

Conclusion: Our study showed that majority of our breast cancer patients were younger age with large tumor size, lymph node positive and poorly differentiated tumors. Younger age women are more likely to present with larger tumor size as compared to older age groups. EGFR expression was most likely associated with ER and PR negative tumors. Assessment of multiple molecular markers aids to understand the biological behavior disease prognosis in Ethiopian population. It might also help to predict which group of patients might get more benefit from the selected treatment strategies and which are not.

Key words: Breast cancer, ER, PR, EGFR, VEGF-A, IHC, Histopathological features

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Acronyms

AJCC	American Joint Committee on Cancer
DAB	3,3′-diaminobenzidine
EGF	Epidermal Growth Factor
ER	Estrogen Receptor
EGFR	Epidermal growth factor receptor
FFPE	Formalin-fixed paraffin-embedded
FISH	Fluorescence in situ hybridization
HER2	Human Epidermal Growth Factor (EGF) Receptor-2
H & E	Hematoxylin-Eosin
HRP	Horseradish peroxidase
IHC	Immunohistochemical
IDC	Invasive Ductal Carcinoma
ILC	Invasive Lobular Carcinoma
MRM	Modified Radical Mastectomy
PR	Progesterone Receptor
SPMMC	St. Paul Millenium Medical College
TASH	Tikur Anbesa Specialized Hospital
TGFα	Transforming growth factor-alpha
TNBC	Triple Negative Breast Cancer
VEGF	Vascular Endothelial Growth Factor
WHO	World Health Organization

CHAPTER ONE

1. INTRODUCTION

1.1. General Overview

The growth, development and function of mammary gland are under the control of signaling pathways influenced by hormonal and growth factors (Oka, et al. 1991). Similar mechanisms are also involved in the development of cancer (Hynes and Watson 2010; LaMarca and Rosen 2008; Sting 2011). Generally, these factors exert their actions via their particular receptors among which some are documented as key biomarkers for breast cancer diagnosis and treatment (Dai, et al. 2016).

Breast cancer belongs to types of malignant tumors that originate in the ductal or lobular region of the mammary gland (Benson, et al. 2009). From the different types of cancer, it is the most frequent and major cause of death among women worldwide (Bray, et al. 2018).

Although breast cancer comprises deregulated cell growth, it has been well known to be a heterogeneous disease showing major genotypic and phenotypic variations (Vaz-Luis, et al. 2012; Weiner, et al. 2012). These variations can be characterized by differences in clinical behavior, morphological appearances and genetic expressions (Penault-Llorca and Viale 2012). Breast cancer molecular subtypes identified on the basis of gene expression profiles underlie the biological behavior of the tumors which differ in prognosis (Salhia, et al. 2011). The differences in molecular characteristics can also affect sensitivity to chemotherapy (Andre, et al. 2006).

Gene expression profiling is not commonly used for retrospective studies due to its high cost, type of required specimen, use of complex technique (Rakha, et al. 2009; Tang, et al. 2008). In order to overcome such problems, it has been substituted with clinically important, cost

effective, technically feasible and readily available immunohistochemical (IHC) techniques (Sisti et al., 2016; Tang et al. 2008).

Among the clinico-morphological variations, tumor size, tumor grade, involvement of axillary lymph nodes and histologic types are used as conventional prognostic factors (Gilarranz 2012). Moreover, estrogen receptor (ER), progesterone receptor (PR) and Human Epidermal Growth Factor Receptor-2 (HER2) are central biomarkers used for molecular classification and also well documented as standard prognostic molecular biomarkers in breast cancer (Taneja, et al. 2010). These biomarkers are also used as therapeutic targets and predictive factors in breast cancer treatment althogh not all cancer cases benefit from the therapy due to intrinsic and acquired resistances (Osborne and Schiff 2011; Rexer and Arteaga 2012).

Apart from the standard biomarkers, aggressiveness, survival and treatment response is influenced by other molecular factors such as angiogenic factors and various growth factor receptors. Epidermal growth factor receptor (EGFR) and vascular endothelial growth factor (VEGF) are emerging breast cancer biomarkers which play important role in tumor growth, metastasis, patient survival and response to treatment (Wahid, et al. 2017). Hence, the study of both standard and emerging molecular biomarkers in breast cancer together with the histopathologic features helps to understand the tumor characteristics and selection of appropriate treatment.

Although assessments of molecular features of the tumor are critical, diagnosis and management of breast cancer in Ethiopian population have been mainly dependent on clinico-pathological features due to inadequate facilities. Moreover, the clinical, pathological and standard biomarker studies are limited. So far, no breast cancer study report is available regarding the EGFR and VEGF expression statuses among Ethiopian breast cancer cases.

1.2. Literatures Review

1.2.1. Epidemiology of Breast Cancer

Globally, cancer has become a main health problem. It was estimated to increase from 14.1 million in 2012 to 18.1 million new cases in 2018 and as a cause of death from 8.2 million in 2012 to 9.6 million people in 2018 (Bray, et al. 2018; Ferlay, et al. 2015). Based on meta-analysis result obtained from 22 countries between 2000 and 2015, the overall incidence of female breast cancer in Africa was estimated to be 24.5 while in East Africa it was somewhat increased to 28.0 per 100,000 people in 2015 per year (Adeloye, et al. 2018). On the other hand, the age-standardized incidence and mortality rate among Eastern African women in 2018 was estimated to be 29.9 and 15.4 respetively (Bray, et al. 2018).

According to the Addis Ababa Cancer registry (AACR) report between 2012 and 2015, breast cancer was ranked first (33%) among the 5,920 female cancer cases. Moreover, based on this data, the incidence of cancer in Ethiopia among men and women was estimated to be 21,563 and 42,722 respectively in 2015. Among these, colorectal cancer was the most common in men (12.21%) while breast cancer was the most common type of cancer (32.74 %) in females followed by cervical (14.15%) and ovarian cancer (5.7%). Additional data obtained from five regional university hospital pathology reports in 2015 revealed that breast cancer was the leading cause (23%) of cancer among women followed by cervical cancer (20%) (Memirie, et al. 2018). Woldu et al. (2017) showed breast, uterine, colorectal, gastric, cervical and esophageal cancers were ranked from first to fifth types of cancers presented to Tikur Anbesa Specialized Hospital (TASH) cancer center from November, 2015 to June, 2016. Among these, breast and uterine cancers were presented to be the most common types in females. In 2018, the

incidence of breast cancer was still estimated to be the most frequent type of cancer among Ethiopian women (Figure 1).

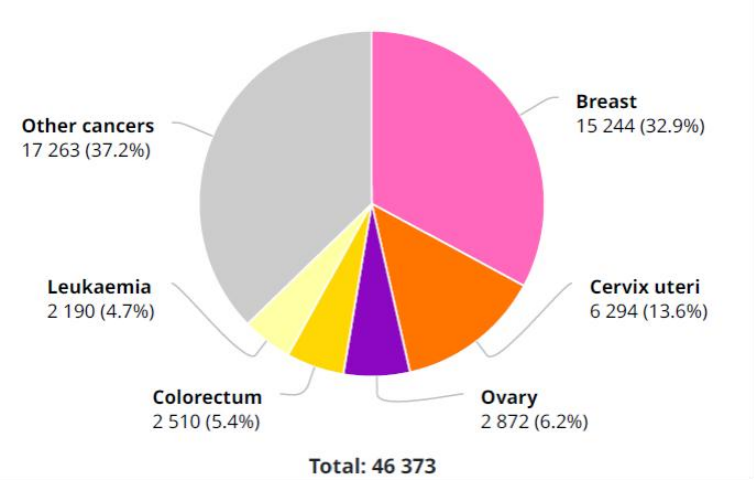

Figure 1. Estimated incidence of Ethiopian female cancer cases of all ages in 2018 (Bray, et al. 2018**).**

According to World Health Organization (WHO 2014) breast cancer was the highest cause of mortality in 2014 among all types of cancer for Ethiopian females (Figure 2).

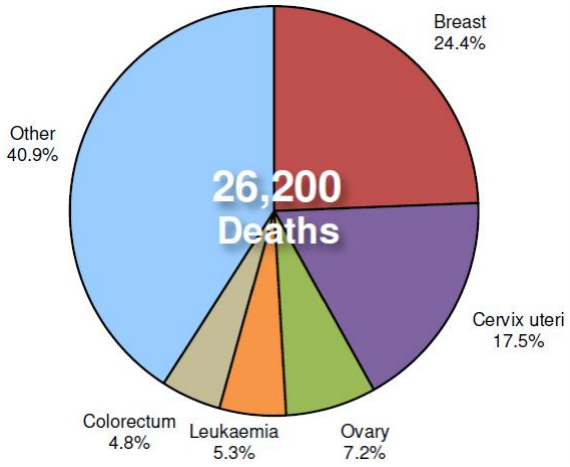

Figure 2. Percentage distributions of mortality among female cancer cases in Ethiopia in 2014. (WHO 2014).

1.2.2.Breast Histology

Before adolescence, the anatomical structure of human breast in males and females is similar. Until then, the small duct is enclosed in stroma and fat cells. During adolescence period in females, anatomical changes such as development of lobules occur as a result of changes in production of sex hormones. Production of sex hormones also varies during menses, breast feeding and change in menopausal status (Gusterson and Stein 2012). Functionally, the mammary gland mainly comprises the larger branched form of ductal system which end up terminally with bunches of ducto-lobular structures. After child birth, the lobules and terminal part of the ducts contain glands and secret milk that is delivered via the ducts to the nipple (Javed and Lteif 2013; Macias and Hinck 2012).

Structurally, adult female breast is composed of two parts, namely, epithelial and mesenchymal portions. The epithelial portion consists of luminal and myoepithelial (basal) cell layers. The inner part of lobules and ducts are formed by layers of the luminal epithelial cells surrounded by the contractile myoepithelial cell layers and the basement membrane (Gusterson and Stein 2012) (Figure 3). In addition, the mammary stem cells are possibly located in both cell layers and can generate both luminal and myoepithelial cells through different lineages (Gusterson, et al. 2005; Visvader 2009). The remaining spaces between the lobules and ducts are filled with fibrovascular mesenchymal tissues composed of blood and lymphatic vessels, fatty tissues, fibroblasts and stroma (Lynch, et al. 2006; Russo and Russo 2004). The three types of cells (luminal, myoepithelial and mammary stem cells) can be distinguished by immunohistochemical determination of the types of protein they express although there are some overlaps in some of the expressions. For instance, luminal cells express cytokeratins 8, 18

and 19 whereas myoepithelial cells express cytokeratins 5, 6, 14 and 10 in addition to other proteins (Bertucci, et al. 2012; Nakshatri, et al. 2009).

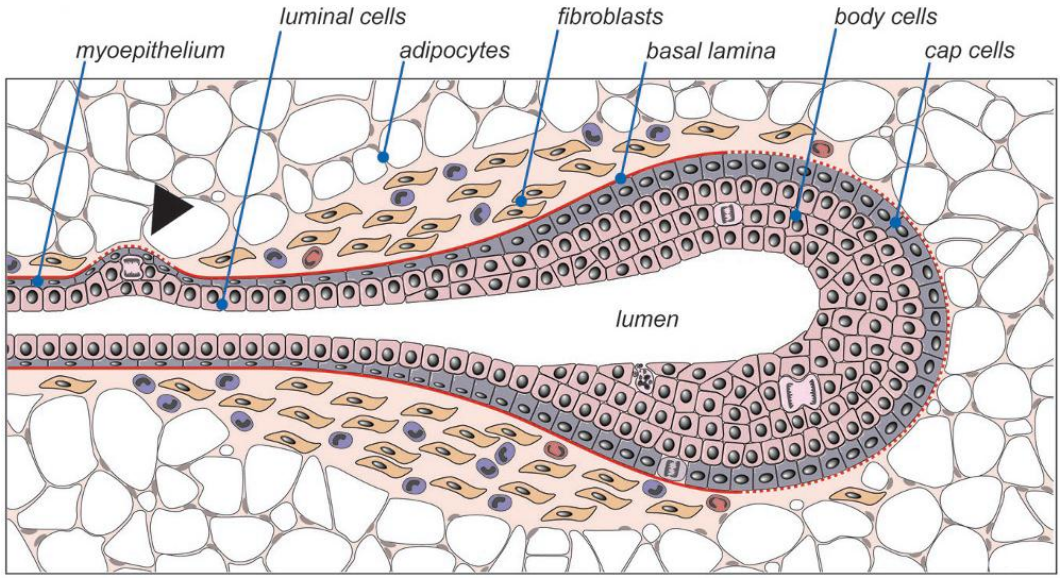

Figure 3. Schematic representation of the ducto-lobular end of mammary gland. Adapted from Sternlicht (2006).

1.2.3. Hallmarks of Cancer

Each type of cancer is characterized by its own genomic organization and protein expressions. Even within each type of cancer, the tumor heterogeneity has been observed (Hanahan and Weinberg 2000). Transformation to cancerous cell requires six hallmarks' to be developed by the normal cells during evolvement of all types of cancer as suggested by Hanahan and Weinberg in 2000. In 2011, these hallmarks were updated with the emerging of two more suggested hall marks and two enabling characteristics (Hanahan and Weinberg 2011) (Figure 4).

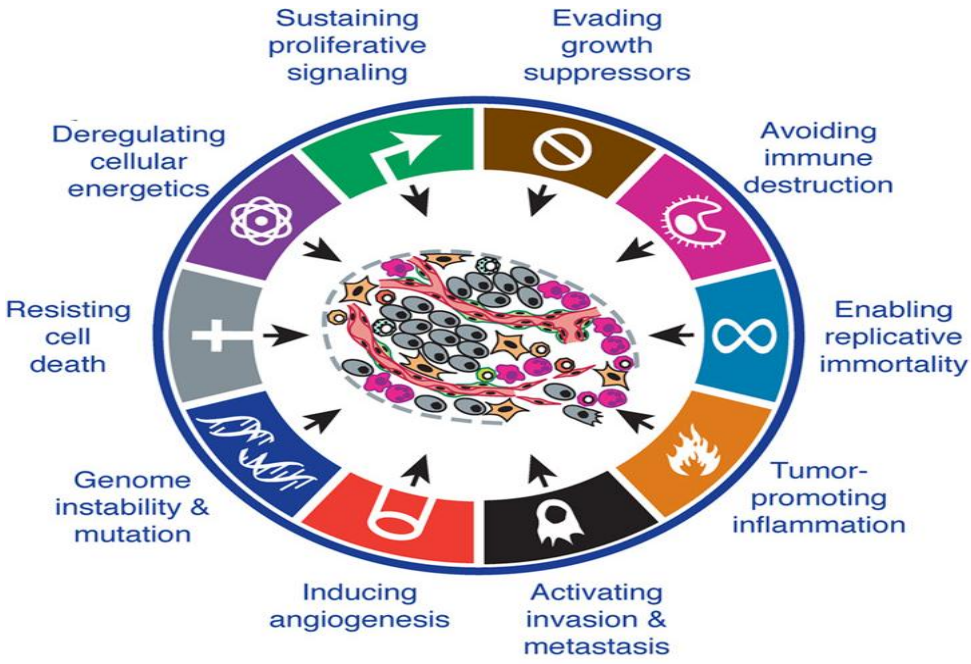

Figure 4. Hall marks of cancer. Tumor-promoting inflammation and genomic instability and mutation are regarded as enabling characteristics. Adapted from Hanahan and Weinberg (2011).

1.2.4. Prognostic and Predictive Factors of Breast Cancer

1.2.4.1 Classical Prognostic Factors

The clinicopathological features including histological type and grade, tumor size, lymph node status, lymphovascular invasion and age of the patient belong to conventional prognostic factors which are well documented and routinely used in invasive breast cancer (Ly et al. 2012; Weigel and Dowsett 2010).

Histologic type

The usual classification system of breast cancer is based on their histological appearance and pattern of growth. Breast cancer can generally be classified into ductal or lobular carcinoma based on the origin from where the cancer arises. Furthermore, it can be categorized into

invasive and non-invasive whether they invade the surrounding tissue or not. Different histological subtypes of invasive breast cancers that have been known are listed in Table 1 (Sinn and Kreipeb 2013).

The commonest histological type of breast carcinoma is invasive ductal carcinoma-not otherwise specified (IDC-NOS) which accounts more than 75% of breast cancer cases (Fulford et al., 2006). It is called 'NOS' (sometimes called NST, no special type) because of its heterogeneity and not further defined by specific histolopathologic features (Sinn and Kreipeb 2013; Weigelt, et al. 2010). Next to IDC-NOS, invasive lobular carcinoma (ILC) was shown to be the abundant histologic subtype comprising up to 15% of the breast cancer cases (Reed, et al. 2015). The other types of invasive breast cancer types accounts for less than 5% of the breast cancer frequency. These include medullary, mucinious, tubular, cribriform, apocrine, invasive micropapillary, metaplastic and other rare histologic subtypes of breast carcinoma (Li, 2010; Rakha and Ellis 2011; Sinn and Kreipeb 2013).

Non-invasive breast cancer types are mainly classified into two types, namely, ductal carcinoma in situ (DCIS) and lobular carcinoma in situ (LCIS). In the case of DCIS, the cancer cells are found within the normal epithelial cells of the duct. Similarly in LCIS, cancer cells are found within the lobules. Both are non-invasive because they are situated ether in the duct or lobules without invasion to the surrounding tissues. Similarly to the invasive type, DCIS can be subclassified into solid form, papillary, micropappilary and cribriform. DCIS is more common than LCIS and considered as early stage since it has a tendency to develop into invasive type (Virnig, et al. 2010). On the other hand, the relative risk of developing invasive breast cancer for LCIS and DCIS have been estimated to be 7-9 and 4-12 fold in the general population (Sgroi 2010).

Table 1. Invasive breast carcinomas based on WHO Classification of breast tumors (Modified from Sinn and Kreipe 2013)

Histologic Type	**Code**	**Remark**
Invasive Breast Carcinoma of No Special Type (NST), Previously Known as IDC (NOS)	8500/3	It comprises all tumors without the specific differentiating features that characterize the other categories of breast cancers.
Carcinomas of mixed type		Designated as mixed invasive NST and special type or mixed invasive NST and lobular carcinoma. Contain tumors of at least 50% with specialized pattern and also 10% - 49% of a non-specialized pattern.
Invasive lobular, ILC	8520/3	Comprise variants such as classic, solid, alveolar, pleomorphic, tubulolobular, and mixed lobular types.
Tubular Carcinoma and Invasive cribriform carcinoma	8211/3 and 8201/3	They are carcinomas with a particularly favorable prognosis and similar low-grade tumor nuclear features.
Invasive mucinous carcinoma	8480/3	Characterized by luminal mucinous A molecular subtype but, those presenting with large cell clusters as hypercellular or type B mucinous carcinomas show a gene expression pattern that is similar to that of neuroendocrine carcinomas.
Carcinomas with medullary features	8510/3 8513/3 8500/3	They are an overlapping group of tumors with more or less 'medullary' appearance, and are described separately according to the new WHO classification.
Carcinomas with apocrine differenciation/ overlaps with Apocrine carcinoma		Mostly comprise tumors that are otherwise of no specific type; however, apocrine differentiation is also seen in special-type carcinomas
Carcinomas with signet-ring cell differentiation		Described together with mucinous carcinoma in the new WHO classification.
Invasive micropapillary carcinoma	8507/3	A luminal-type breast cancer with a propensity for lymphovascular invasion and regional lymph-node metastasis.
Metaplastic carcinoma of no special type	8575/3	Represents a group of unrelated invasive breast cancers displaying differentiation of the tumor cells into squamous or mesenchymal-looking elements.
Carcinomas with neuroendocrine features (neuroendocrine tumor, well differenciated)	8574/3	Mostly they have low- or intermediate-grade nuclear features, and morphological features similar to those of neuroendocrine tumors of the gastrointestinal tract and of the lung.
Adenoid cystic carcinoma	8200/3	The most frequently encountered salivary-type tumor of the breast and is, in the great majority of cases, a low-grade malignant tumor
Invasive papillary carcinoma	8503/3	Specially differentiated adenocarcinoma of the breast with papillary morphology, but otherwise no distinguishing clinical, genetic, or prognostic features.

Tumor grade

Although classification by histologic type of breast cancer is important, similar histologic types, especially in the case of IDC, exhibits significant variation in their degree of tumor cell differentiation (tumor histologic grade) and other biological features (Makki, et al. 2015). Histologic grade has been well documented to be one of the strongest predictor of patient outcome in invasive type of breast cancer. Various studies have shown the correlation between patient survival and degree of breast tumor differentiation (Rakha, et al., 2008; Stankov, et al.2012; Zhu et al., 2017). Moreover, positive association between risk of disease recurrence and tumor grade in breast cancer (Aleskandarany, et al. 2011).

Determination of tumor grade is based on the degree of nuclear pleomorphism, glandular differenciation and mitotic frequency. Hence it provides information regarding the aggressive nature of the tumor (Arpino, et al., 2015; Rakha et al., 2010). The most widely used and currently recommended method for histological grade evaluation is the Nottingham combined histological grade (also called the Nottingham or the Elston-Ellis modification of the Scarff-Bloom-Richardson method) (Elston and Ellis 1991; Frkovic-Grazio and Bracko 2002; Zhang, et al. 2010) (Table 2). Based on this classification, the tumor grade is usually sub-classified into three types, namely, grade 1 or well differenciated, grade 2 or moderately differenciated, and grade 3 or poorly differenciated tumors. Poorly differenciated tumors are the most aggressive while well differenciated tumors are the least aggressive tumor types (Atanda et al., 2017; Rakha et al. 2010).

Table 2. Nottingham combined histological grading system for invasive breast cancers (Elston and Ellis 1991)

Feature	Criteria	Score
Tubule formation	Majority of tumor (>75%)	1
	Moderate degree (10-75%)	2
	Little or none (<10%)	3
Nuclear pleomorphism	Small, regular uniform cells	1
	Moderate increase in size and variability	2
	Marked variation	3
Mitotic counts (/10 high‑power fields)	0-5 6-10 >11	1 2 3
Overall tumor grade	3-5 points 6-7 points 8-9 points	Grade 1 – well-differentiated Grade 2 – moderately differentiated Grade 3 – poorly differentiated

Tumor size

The size of the tumor is another potent prognostic factor in female breast cancer. The maximum diameter of the tumor determined from the pathologic sample is considered to be the pathologic size of the tumor (Soerjomataram, et al. 2008). Pathologic tumor size is generally classified into four categories (Giuliano, et al. 2017) (Table 3). Studies have indicated that worse clinical outcome was observed in female breast cancer with larger tumor size (Chia, et al. 2004; Mansano-Schlosser, et al. 2017; Zheng, et al. 2015a).

Table 3. Pathological Tumor Size Classification Based on AJCC Definition (Giuliano, et al. 2017)

T Category	T Criteria
pTx	Primary tumor cannot be assessed
pT0	No evidence of primary tumor
pTis (DCIS)	Ductal carcinoma in situ (DCIS)
pTis (Paget)	Paget disease of the nipple NOT associated with invasive carcinoma and/or carcinoma in situ (DCIS) in the underlying breast parenchyma. Carcinomas in the breast parenchyma associated with Paget disease are categorized based on the size and characteristics of the parenchymal disease, although the presence of Paget disease should still be noted.
pT1	Tumor<20mm in greatest dimension
pT2	Tumor>20mm but <50mm in greatest dimension
pT3	Tumor>50mm in greatest dimension
pT4	Tumor of any size with direct extension to the chest wall and/or to the skin (ulceration or macroscopic nodules); invasion of the dermis alone does not qualify as T4

Lymph node involvement

Histologic assessment of axillary lymph node status has been shown to be one of the independent and strongest prognostic factors in female breast cancer (Fitzgibbons, et al. 2000; Morabito, et al. 2003). Whereas, in the case of negative axillary lymph node status, sentinel lymph node biopsy is considered to be valuable diagnostic procedure to confirm the presence of early tumor metastasis to the axillary lymph nodes (Veronesi, et al. 2001). Pathological classification of axillary lymph node status based on American Joint Committee on Cancer (AJCC) is one of the common techniques in breast cancer diagnosis (Giuliano, et al. 2017) (Table 4). The assessment of axillary lymph node and sentinel lymph node status are important

procedures to define the risk of the breast cancer disease (Goldhirsch, et al. 2007). Moreover, evaluating the number of axillary lymph nodes is significant for breast cancer management (Goldhirsch et al., 2009).

Table 4. Pathological Axillary Lymph Node Status Classification Based on AJCC Definition (Giuliano, et al. 2017).

Category	Criteria
pNX	Regional lymph nodes cannot be assessed (e.g., previously removed, or not removed for pathological study)
pN0	No regional lymph node metastasis
pN1	Micrometastases; or metastases in 1 to 3 axillary ipsilateral lymph nodes; and/or in internal mammary nodes with metastases detected by sentinel lymph node biopsy but not clinically detected
pN2	Metastasis in 4–9 ipsilateral axillary lymph nodes, or in clinically detected* ipsilateral internal mammary lymph node(s) in the absence of axillary lymph node metastasis
pN3	pN3a = Metastasis in 10 or more ipsilateral axillary lymph nodes (at least one larger than 2 mm) *or* metastasis in infraclavicular lymph nodes pN3b = Metastasis in clinically detected internal ipsilateral mammary lymph node(s) in the *presence* of positive axillary lymph node(s); or metastasis in more than 3 axillary lymph nodes *and* in internal mammary lymph nodes with microscopic or macroscopic metastasis detected by sentinel lymph node biopsy but not clinically detected pN3c = Metastasis in ipsilateral supraclavicular lymph node(s)

With regard to the combined prognostic factors, the AJCC tumor, node, metastasis (TNM) classification system is commonly used for staging of the tumor at the time of diagnosis and the strongest prognostic factor for breast cancer recurrence. The clinical and pathological staging according to this classification system comprises three characterstics of the tumor, namely, size of the primary tumor (T), axillary lymph node status (N) and presence of metastasis to distant sites (M) and hence the tumor is assigned from stage I to stage IV (Table 5).

Table 5. AJCC TNM Anatomic Stage Groups (Giuliano, et al. 2017)

T Category	N Category	M Category	Stage Group
Tis	N0	M0	0
T1	N0	M0	IA
T0	N1mi	M0	IB
T1	N1mi	M0	IB
T0	N1	M0	IIA
T1	N1	M0	IIA
T2	N0	M0	IIA
T2	N1	M0	IIB
T3	N0	M0	IIB
T1	N2	M0	IIIA
T2	N2	M0	IIIA
T3	N1	M0	IIIA
T3	N2	M0	IIIA
T4	N0	M0	IIIB
T4	N1	M0	IIIB
T4	N2	M0	IIIB
Any T	N3	M0	IIIC
Any T	Any N	M1	IV

Age, race and ethnicity

Age at diagnosis is shown to be one of the important prognostic factors in female breast cancer. Especially occurrence of breast cancer at younger age was shown to be related to aggressive

behaviour of the tumor and poor prognosis (Brandt, et al. 2015; Han, et al. 2004). Hence, age at diagnosis has been suggested to be used to as a prognostic marker to determine which groups of patients are at high risk (Goldhirsch, et al. 2005). Although it is not well-established, race and ethnicity has been suggested to be one of the prognostic factors because various studies indicated that higher histological grade and poor prognosis was shown more in breast tumors of African origin than Caucasians (Curtis, et al. 2008; Henson, et al. 2003; Newman, et al. 2006).

1.2.4.2. Standard Prognostic and Predictive Factors

The well-known prognostic as well as predictive factors most widely used for invasive breast cancer include expression status of ER, PR and HER2 (Yadav, et al. 2016). Both prognostic and predictive factors play central role to determine the disease prognosis and selection of appropriate treatment plan (Tonini, et al. 2008).

Estrogen receptor

Estrogen and progesterone receptors are members of steroid hormone receptor family that belong to nuclear receptor superfamily. Once activated, they can translocate to the nucleus and control gene expression by binding to their DNA target. Although assessment of both receptors is important to determine respnoses against endocrine therapies, emphasis has been given to ER since the PR expression is commonly depends on ER expression in the mammary gland (Hennighausen and Robinson 2005; Wierman, 2007; Yaşar, et al. 2017).

There are two major types of ER, namely, ERα and ERβ that are expressed from two independent ER genes, ESR1 (located on chromosome 6q24–27) and ESR2 (located on chromosome 14q21-22) respectively. The wild types of both isoforms share nearly 96% homology in the DNA binding and 58% homology in their hormone receptor binding domain at the receptor level (Higa and Fell 2013; Yasar, et al. 2017) (Figure 5). Both of the receptor

isoforms were revealed to be expressed in the breast although there was variation in the site of expressions. ERα was detected mainly in the nucleus of luminal epithelial cells while ERβ expression was seen in luminal epithelium, myoepithelial cells and in the stroma (Speirs, et al. 2002). Although both isoforms have similar ligand-binding affinity, ERβ is the major type of isoform expressed by the inactive mammary epithelial cells while ERα is expressed only in 10-20% of the cells (Leung, et al. 2012).

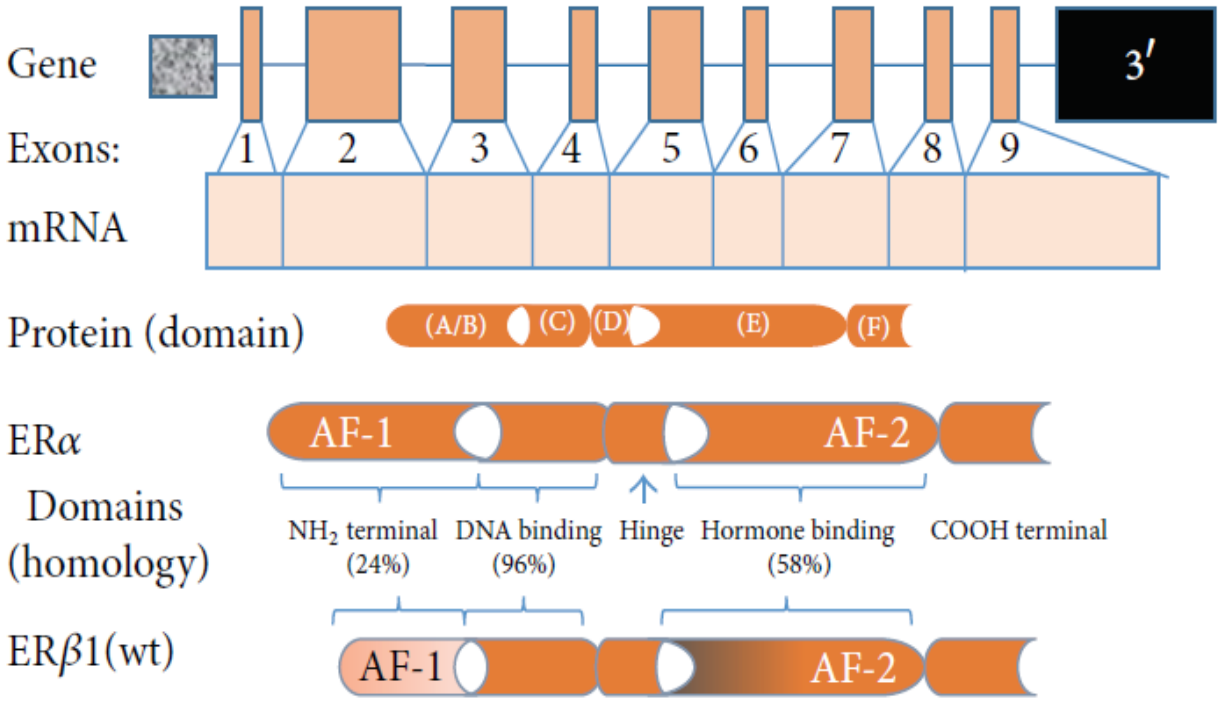

Figure 5. Schematic representation of ER gene and protein structure. The protein domains include NH2-terminal domains (A/B) containing activating factor (AF)-1, central region (C), hinge domain (D), multifunctional (hormone binding) domain (E) containing AF-2 and the carboxyl-terminal domain (F). Adapted from Higa and Fell (2013).

The epithelial cell growth in the mammary gland is influenced by estrogen during pregnancy, lactation as well as menstrual cycle. Gene knockout studies revealed that ERα is important for the development of normal duct while ERβ has a major role in differentiation of the ducts to alveoli which can secrete milk (Feng et al., 2007; Forster, et al. 2002). In other studies, ERβ has

been signified to have both proliferative and anti-proliferative activity in normal cells of the mammary gland (Cheng, et al. 2004, Helguero, et al. 2005).

Apart from its central role in the growth of the normal mammary gland, various studies have shown that ERα has important role in breast cancer development and progression via various classical and nonclassical signaling pathways (Martin, et al. 2014; Murphy and Leygue 2012) (Figure 6). The expression of ERα has been shown to be common in breast cancer cases (Esslimani-Sahla, et al. 2005; Park, et al. 2003). Various studies revealed that ERα positive tumors enhance progression of cancer whereas ERβ suppresses the transcriptional activation of ERα when treated with estrogen (Hartman, et al. 2006; Nilsson, et al. 2001). ERβ was also shown to induce cell cycle arrest and hence inhibit cell proliferation and tumorigenesis (Paruthiyil, et al. 2004). Other studies reveled that ERβ enhance proliferative and invasive ability of breast cancer cell lines (Hou, et al. 2004; Tonetti, et al. 2003).

ERα positive tumors are generally characterized by lower grade, better prognosis, longer disease free survival and responsive to hormonal treatment, radio and chemotherapy (Forster, et al. 2002). On the other hand, ERα negative breast cancer cases commonly present with higher grade, shorter survival and resistant to tamoxifen and radio-therapy (Forster, et al. 2002; Hennighausen and Robinson 2005).

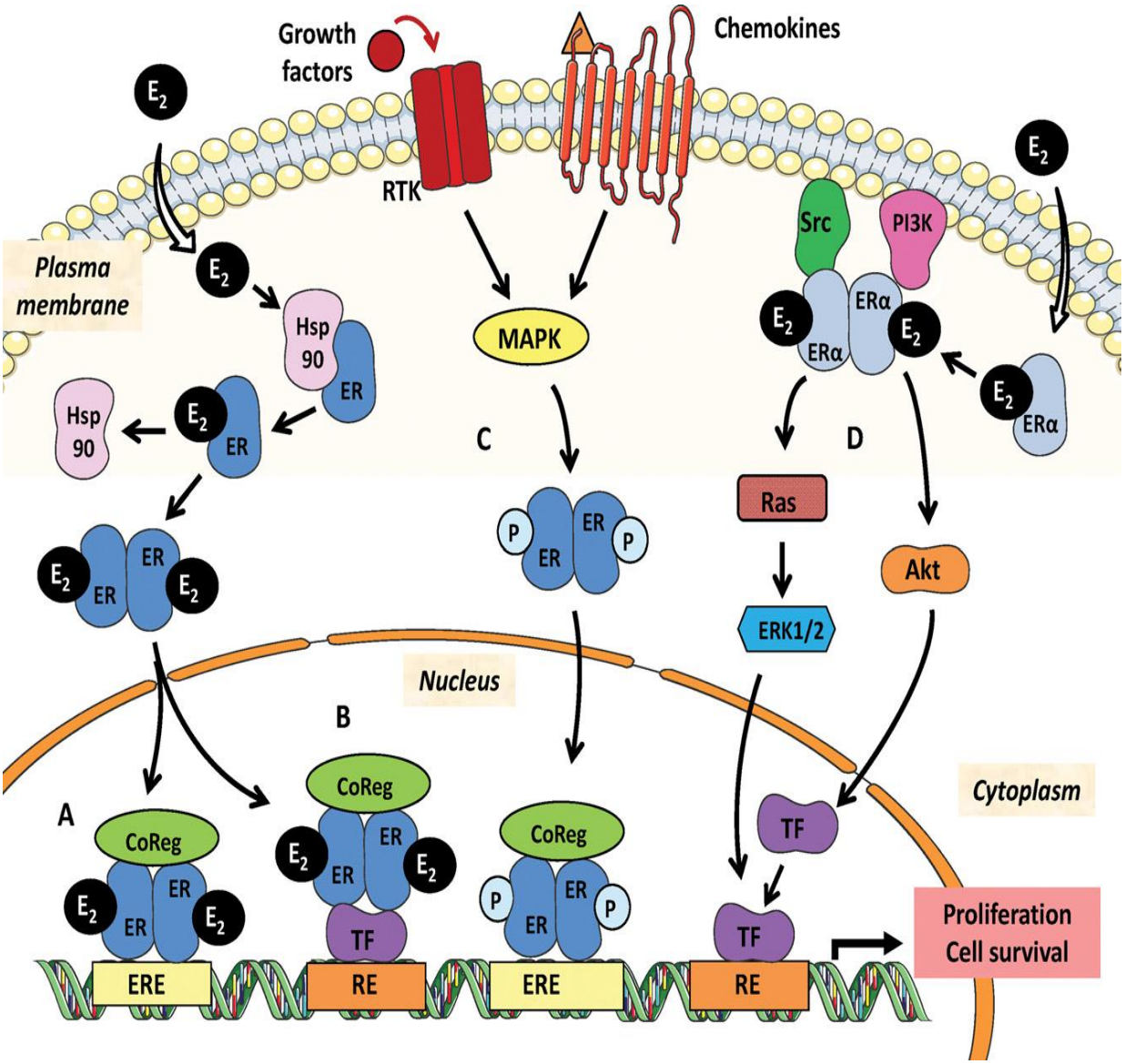

Figure 6. The classical and nonclassical ER signaling pathways: A) Classical genomic pathway; B) nonclassical genomic pathway; C) Ligand-independent growth factor-induced ER activation; D) nongenomic pathway. ER represents the common pathways for ERα and ERβ unless specified. Abbreviations: CoReg, coregulator; E2, estrogen; ERE, estrogen response element;Hsp90, heat shock protein 90; MAPK, mitogen activated protein kinase; PI3K, phosphatidylinositide 3-kinase; P, phosphorylation; RE, Response element to other transcription factors; RTK, receptor tyrosine kinase; Src, Sarcoma virus oncogene; TF, transcription factor. Adapted from Le Romancer, et al. (2011).

Detection of the ER is important to predict tumor response against hormonal therapy. Both receptors are dependent on each other but ER status is regarded at present as the most powerful predictive marker in breast cancer management (Mohsin, et al. 2004; Payne, et al. 2008). Moreover, majority of the primary invasive breast tumors express this receptor. Hence, this receptor becomes one of the main targets in breast cancer treatment (Yersal and Barutca 2014).

Currently, different therapeutic methods are in use to inhibit the activity of ER. Selective ER modulators such as tamoxifen block the ER activity in the breast. Tamoxifen is a selective ERα-binding antagonist which can be given as an adjuvant therapy for five years (Hennighausen and Robinson 2005; Pistelli, et al. 2018). Drugs such as Fulvestrant are completely antagonist which bind to ER and block its activation and hence accelerate its degradation. Aromatase inhibitors such as Anastozole also used to inhibit aromatase enzyme to reduce the synthesis of estrogen (Blackburn, et al. 2018; Pistelli, et al. 2018). Although there are different anti-estrogen treatment options, the efficacy of these regimens do not exceed 70% as shown in neoadjuvant settings (Colleoni and Montagna, 2012; Miller et al. 2009).

Progesterone Receptor

Progesterone receptors, like ERs, belong to subfamily of nuclear steroid hormone receptors that act as transcription factors upon activation by their ligands (Lange 2004). Mostly, PR expression is transcriptionaly regulated by estrogen-activated ERα and hence its expression indicates the functional downstream signaling of ER (Diep, et al., 2015; Ikeda, et al. 2015; Williams, et al. 2008).

Under normal condition, only 20-30% of the mammary luminal cells express PR. During development and expansion of the mammary gland, especially in adults, these cells undergo proliferation via autocrine manner (Hilton, et al. 2015; Obr and Edwards 2012). Moreover, the

PR+ epithelial cells also promote cell proliferation via paracrine pathways. In this mechanism, the PR+ cells induce expression and secretion of various mitogens which act on nearby PR-negative cells to proliferate (Lydon and Edwards 2009; Tanos, et al., 2012).

Unlike the ER isoforms, which are transcribed from different genes, three PR isoforms (PR-A, PR-B and PR-C) originate from the same gene (located at chromosome 11q22.1) (Chen, et al. 2008). Structurally, both PR-A and PR-B isoforms have amino terminal domain (NTD) containing transcriptional activation function (AF) domains, DNA binding domain (DBD), hinge region (H), and a C-terminal hormone-binding domain (HBD) (Hill, et al. 2012; Sherbet, 2017) (Figure 7). While PR-B isoform is a full-length form containing three transactivation domains, PR-A is N-terminally truncated isoform lacking 164 amino acid residues and hence it contain only two transactivation function domains. On the other hand, PR-C can't bind to DNA since it contains only hinge region and HBD (Scarpin, et al. 2009).

PR-A and PR-B are commonly expressed functional isoforms upon activation by ligand, whereas, PR-C is non-functional form that usually act as legand-sequester and its expression is limited (Condon, et al. 2006; Mendelson and Hardy 2006). Although both PR-A and PR-B are transcriptionaly active, PR-B is found to be much stronger as compare to PR-A for their target genes (Pathiraja, et al. 2011).

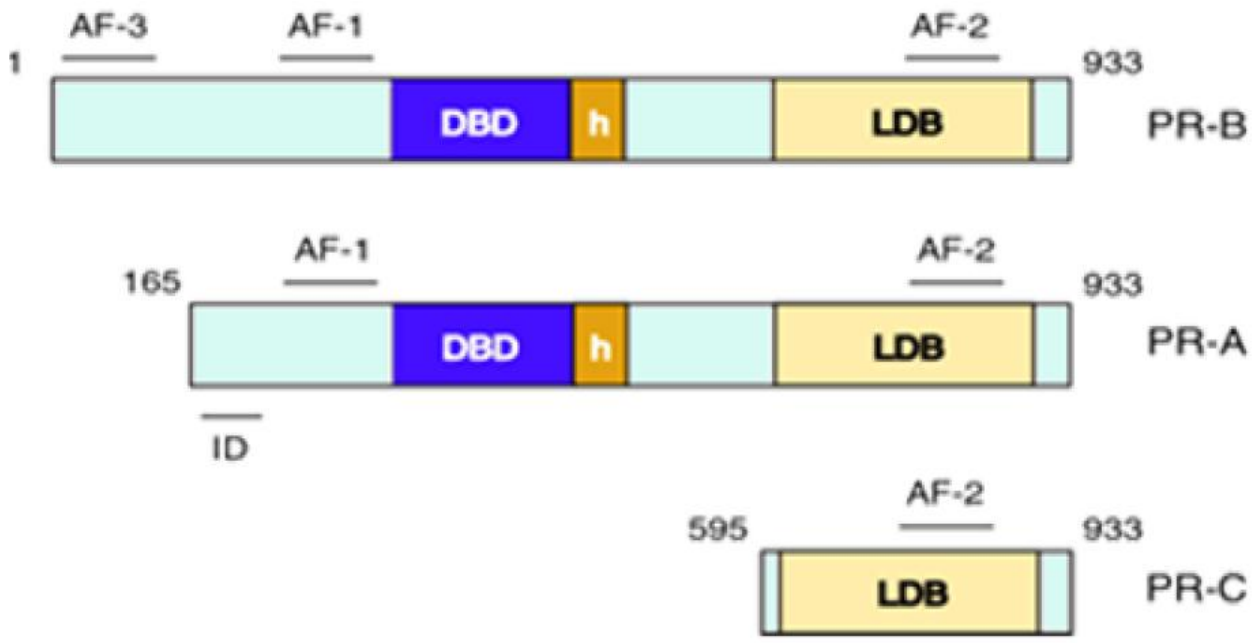

Figure 7. Progesterone receptor isoforms and their domains. Abbreviations: AF: Activation domain; DBD: DNA binding domain; LBD: Ligand binding domain. Adapted from Sherbet (2017).

The PR-biological responses are based on the cell type which in turn depends on the signaling pathways and presence of associated cofactors. Generally, ligand-induced PR-mediated transcriptional activities are accomplished through genomic (direct) and non-genomic (indirect) signaling mechanisms (Figure 8). In the classical genomic pathway, following ligand binding via HBD, both PR-A and PR-B undergo conformational change, homo- or heterodimerization and nuclear translocation (Contrò, et al. 2015). Then, it binds particularly to progesterone response elements (PREs) located at the promoter region and recruit other transcriptional co-activators for target gene expression in the presence of basal transcriptional complexes (Clarke and Graham, 2012; Hagan and Lange 2014). Moreover, PR also modulates gene expression through binding to other transcriptional factors at the non-PRE regions of other genes (Buser, et al. 2007; Clarke and Graham 2012). In the non-genomic (extracellular) pathways, PR also

activates cytosolic MAPK signaling pathways via transactivation of membranous epidermal growth factor receptor. Activation of these kinases in turn leads to activation of several transcription factors including PR by phosphorylation (Hagan, et al. 2012; Hammes and Levin 2007; Lange and Yee 2008).

ER status has been well recognized so far for disease prognosis and predictor for therapeutic response in breast cancer. It has been indicated that PR plays a significant role in breast cancer cell growth and progression via different cytosolic kinase pathways including MAPK and PI3K signaling (Boonyaratanakornkit, et al. 2007; Hagan, et al. 2012). Moreover, the correlations of prognosis and therapeutic response with ER/PR expression have been studied in breast cancer. Various studies reported that ER+/PR+ tumors were associated with better prognosis, decreased risk of recurrence and metastasis, lower mortality rate better response against endocrine therapies as compared to ER+/PR-, ER-/PR- and ER-/PR+ tumors in breast cancer (Dunnwald, et al. 2007; Fallahpour, et al. 2017; Parise and Caggiano 2014; Prata and Peroua, 2011). Hence, PR status plays significant role regarding the prognosis and endocrine therapeutic responses. The PR expression, therefore, suggests its significant role for disease prognosis and prediction of endocrine treatment responses in breast cancer.

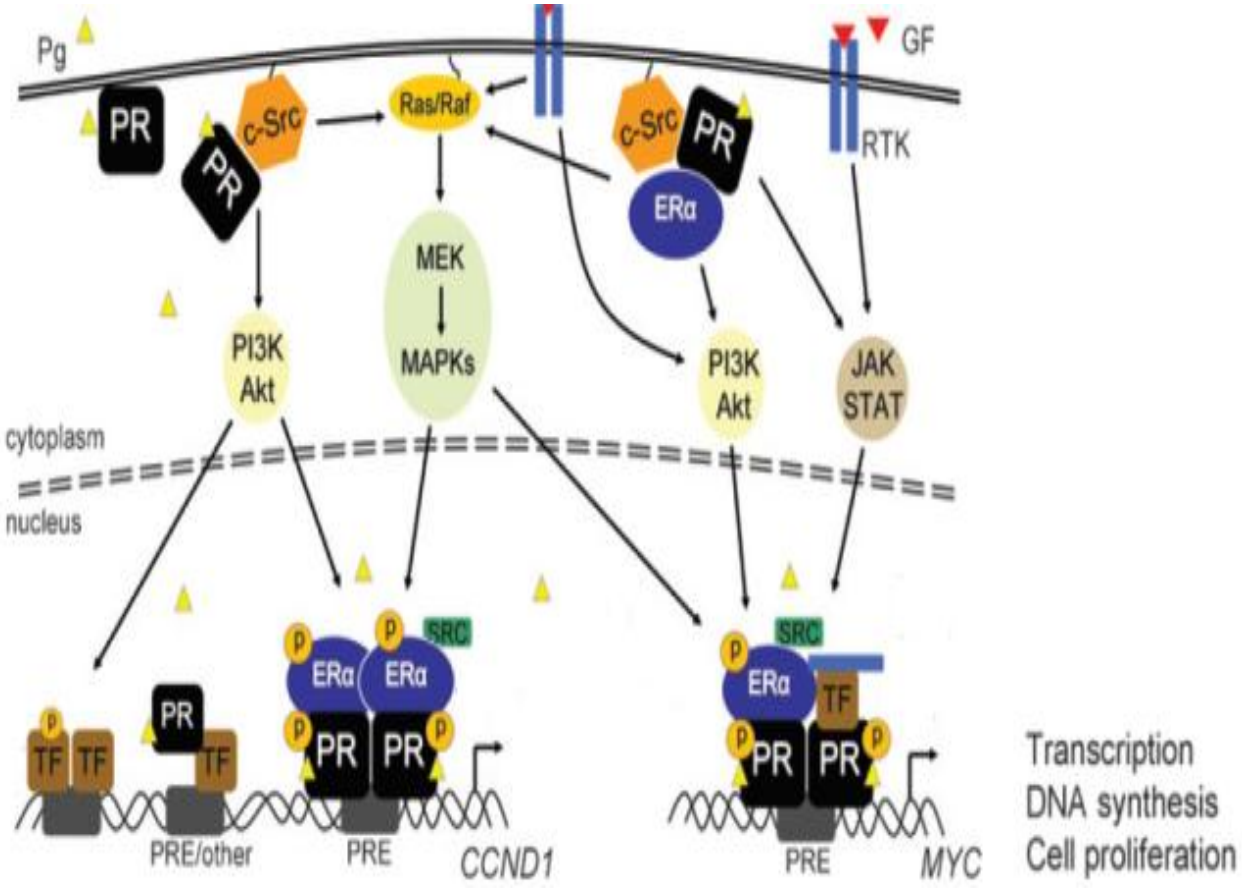

Figure 8. Proposed model of the genomic and non-genomic PR signaling pathways. Abbreviation: CCND1, Cyclin D1. Modified from Giulianelli, et al. (2012).

The role of signaling cross talk between HERs and PR (mainly PR-B) pathways has been studied in breast cancer (Diep, et al. 2015). On one hand, PR has been indicated to play important role in expression of HER ligands and phosphorylation of HERs. On the other hand, HER signaling pathways were shown to regulate transcriptional activity of PR. Since the transcriptional role of PR depends on its phosphorylation status, the kinase effectors of HER family receptors modulate the phosphorylation of PR especially PR-B (Daniel, et al. 2011; Elizalde and Proietti 2012).

Human Epidermal Growth Factor Receptor 2

The other well-known prognostic biomarker used in breast cancer treatment is HER2, a member of EGFR family that also comprises HER1, HER3 and HER4 (Arkhipov et al., 2013; Duffy 2005). Unlike other members of its family, HER2 lacks the ligand binding site but it has the great ability to form heterodimer with other members since it has constitutively active domain. Moreover, HER2 can also form homodimer and initiate its intracellular kinase activity without external stimuli (Burgess, et al. 2003; Emede, et al. 2012) (Figure 9).

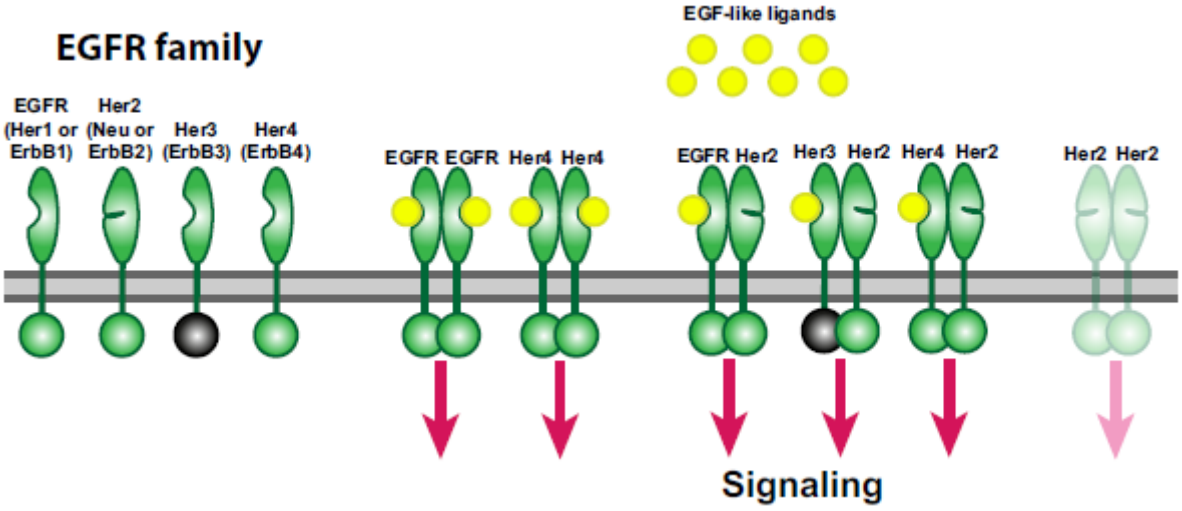

Figure 9. EGFR family members and their possible homo- and heterodimerization. Adapted from Arkhipov et al.(2013).

During signal transduction, HER2 initiates phosphorylation and activation of its intracellular tyrosine kinase domain. This leads to recruitment of signaling molecules which convey the instruction to the nucleus for cell growth, division and DNA repair processes. The signaling also results in other cellular responses based on the type of molecules involved in the signaling process (Vu and Claret 2012) (Figure 10). HER2 has a powerful effect on the cellular processes because it has great kinase activity and signaling networks (Fiszman and Jasnis 2011).

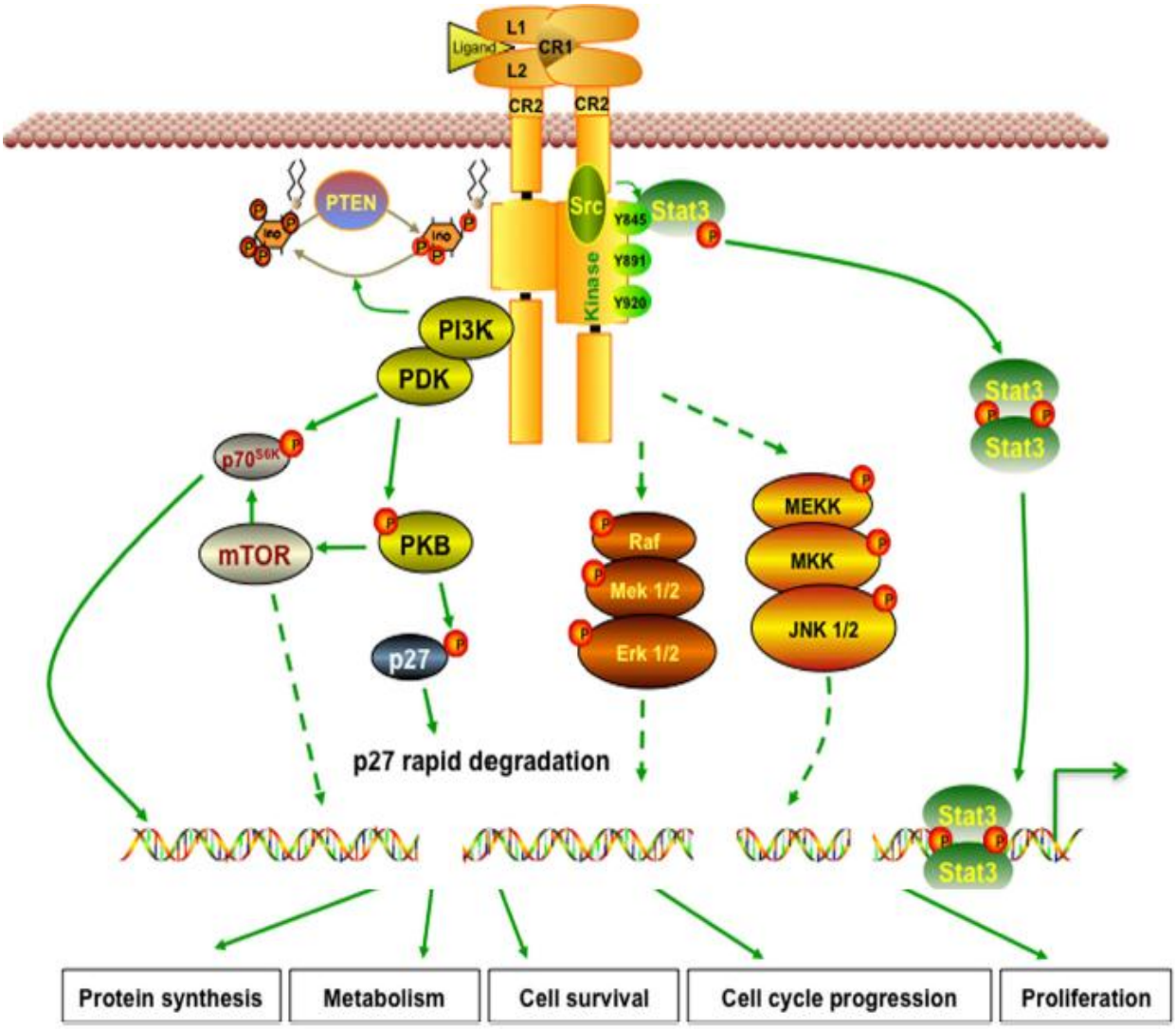

Figure 10. Schematic diagram of HER2 activation and downstream signaling pathways. The solid and broken arrows indicate the major and minor pathways respectively. Abbreviations: CR, Conserved region; Erk, Extracellular signal-regulated kinase; Ino, Inositol; JNK, c-Jun N-terminal kinase; Mek, Mitogen-activated protein kinase/extracellular signal-regulated kinase; MEKK, Mitogen-activated protein kinase (MAPK); MKK, MAPK kinase; mTOR, Mammalian Target of Rapamycin; p27, 27 kDa Cyclin-dependant kinase inhibitor (Kip1); $P70^{S6K}$, p70 ribosomal protein S 6 protein kinase; PDK, Phosphotidylinositide-dependent kinases; PI3K, Phosphatidylinositol 3-kinase; PKB, Protein kinase B (PKB); PTEN, Phosphate and tensin homolog on chromosome ten; Raf, Rapidly accelerated fibrosarcoma; Src, Rous sarcoma oncogene; Stat, Signal transducer and activator of transcription. Modified from Vu and Claret (2012).

The mitogenic potential of HER2 varies with the type of dimers formed and assessed by its proliferative ability. The highest mitogenic ability was shown by HER2-HER3 and HER2-HER1 heterodimers with proliferative index of 10.5 and 9.6 respectively. In breast cancer, such types of heterodimers have been commonly observed. The rest of homo- and hetero dimers have been shown to have lower mitogenic potential. For instance, the mitogenic ability of HER2 homodimeron was found to have a proliferative index of 3.5 (Ghosh, et al. 2011). Hence, heterodimerization with HER2 receptors enables to have greater stability and signaling potential than any type of homo- or heterodimer formed among the HER family members (Emede, et al. 2012; Fiszman and Jasnis 2011).

In many studies, significant but variable proportion of patients overexpressed HER2 in various types of cancer such as breast, ovarian and gastric carcinomas (Browne, et al. 2009; Buday and Downward 1993; Pronk 1993). Overexpression of HER2 mainly arises from gene amplification which in turn, capable to induce carcinogenesis (Negro, et al. 2004). Studies revealed that HER2 gene amplification and overexpression ranges from 15% to 30% in primary breast cancer of invasive type (Duffy 2005).

Assessment of HER2 overexpression could be used to predict positive or negative response against hormonal and systemic therapies such as cyclophosphamide and anthracycline-based therapies in breast cancer at early stage. Although the study for predictive potential of HER2 continues, determination of HER2 status is still used as a prognostic and predictive factor for HER2 targeted therapies (Rosenzweig 2018). HER2 overexpressing tumors have shown poor prognosis and poor response to treatment including hormonal and chemotherapy (Ferretti, et al. 2007).

The differences in molecular expressions among breast cancer was identified through microarray-based gene expression profiling and classified in to four distinct subtypes. These include luminal-like (luminal A and B), HER2 enriched, basal-like and normal-like subtypes (Perou, et al. 2000; Sotiriou, et al. 2003). The luminal A subtype differ from luminal B by higher expression of genes related to ER but lower levels of expression of genes related to cell proliferation (Sorlie, et al. 2001; Yehiely, et al. 2006). The HER2-enriched subtype is characterized by higher level of expressions genes located at HER2 gene but negative for ER or PR expressions (Carey, et al. 2006; Sihto, et al., 2008; Tang, et al. 2009). The basal-like subtypes comprises tumors with lack of HER2 overexpression and low or no expressions of ER and PR but having higher expression of genes related to proliferation as well as expression of genes characterizing the basal/ myoepithelial cells such as cytokeratins and EGFR (Kennecke, et al. 2010). Similar to the basal-like subgroups, normal-like subtypes lacks the expressions of ER, PR and HER2. Moreover, this subgroup has overexpression of various genes that are found in non-epithelial tissues such as adipose tissue and also genes related to basal subgroups. These molecular subtypes have been investigated by IHC surrogates (Cakir, et al. 2012).

1.2.5. Epidermal Growth Factor Receptor (EGFR) and Vascular Endothelial Growth Factor (VEGF) as Potential Prognostic and Predictive Markers

Epidermal growth factor receptor is a classical type of receptor tyrosine kinase that belongs to the HER family. Like other members, EGFR is also involved in the control of various cellular processes under normal or malignant conditions (Wee and Wang 2017).

Structurally, EGFR comprise three major domains having distinct role. These are the N-terminal extracellular ligand binding domain, a transmembrane domain and intracellular domain with tyrosine kinase activity (Wang, et al. 2010). The normal extracellular domain of EGFR comprises four subdomains: subdomain domain I – IV. When there is no ligand, subdomain II interact with subdomain IV to inhibit receptor dimerization. The binding of proper ligand to the subdomains I and III causes conformational change that expose domain II to undergo dimerization (Huang, et al. 2016) (Figure 11). EGFR selectively binds to EGF, transforming growth factor-alpha (TGFα), betacellulin (BTC), amphiregulin (AR), epiregulin (EPR), and heparin-binding EGF (HB-EGF) (Hynes and Lane 2005).

Two types of signaling pathways have been documented regarding EGFR signaling pathways, namely, the classical and the novel type signaling pathways (Figure 12). In the classical pathway, receptor dimerization results in activation and autophosphorylation of the c-terminal tyrosine kinase domain. Phosphorylation at specific positions provides a doking site for various types of adaptor molecules and enzymes that leads to activation of intracellular signaling cascades (Bazley and Gullick 2005; Harari 2004). The novel pathway involves the nuclear transport of EGFR from the cell surface (Brand, et al. 2011; Scaltriti and Baselga 2006). Downstream signaling of EGFR plays a central role in various physiological and pathological

processes such as cellular growth, proliferation and invasion and metastasis (Citri and Yarden 2006).

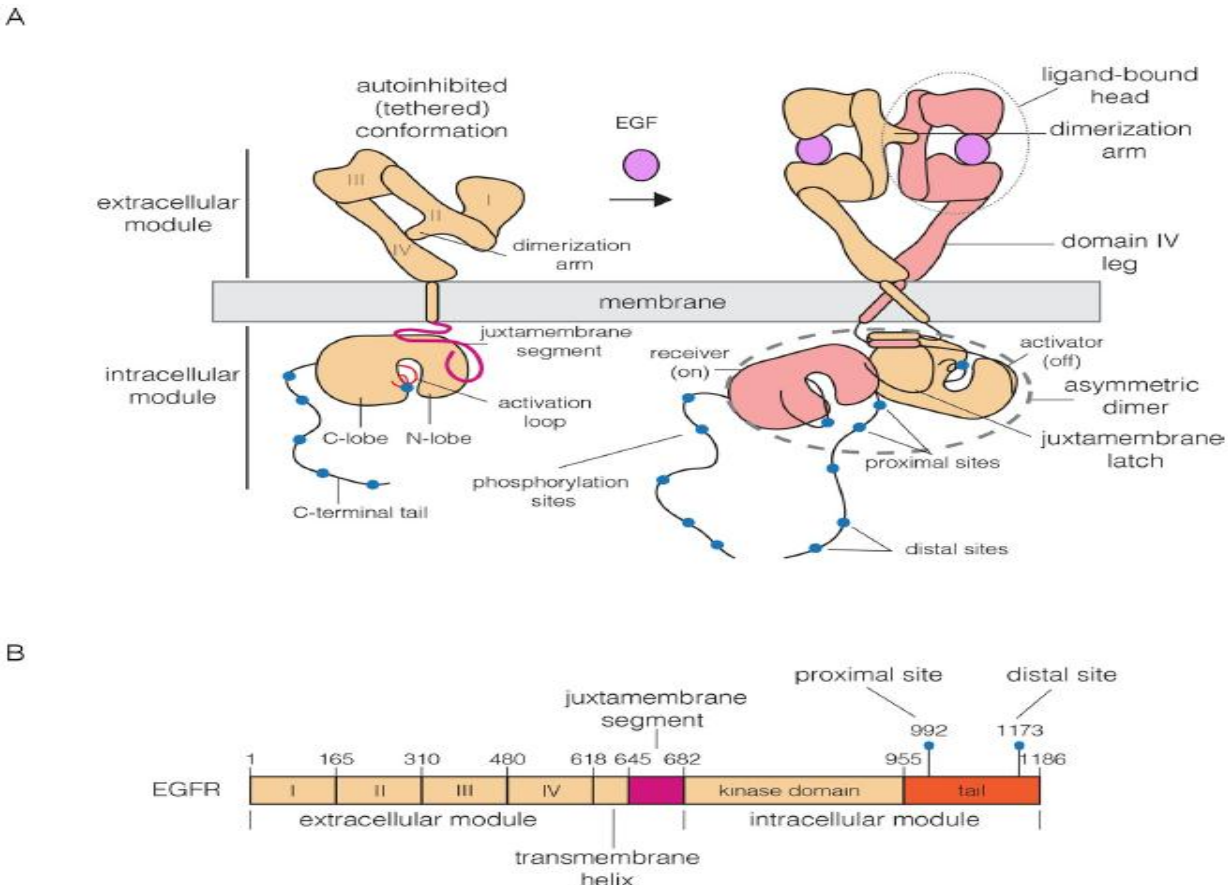

Figure 11. **Schematic illustrations of EGFR structure**. (A) The classical model for transition of monomeric to dimeric form upon ligand binding. Abbreviation: EGF, Epidermal growth facor (B) Human EGFR domain boundaries (24 amino acid residues of the signal sequence are not included). Adapted from Huang et al. (2016).

EGFR plays a significant role in carcinogenesis due to its oncogenic activity via a variety of mechanisms such as overexpression of the receptor and its ligands, defects in its down-regulation, activating mutation and simultaneous expressions of other HER family members. (Hynes and Lane 2005; Tebbutt, et al. 2013). The oncogenic property of overexpressed EGFR ligands was first proven by genetically engineered mouse model overexpressing one of TGF-α

(Sandgren et al., 1990). EGFR expression was associated with poor tumor differenciation, ER negativity, higher mitotic index, poor endocrine treatment response and decreased survival (Arnes, et al. 2009).

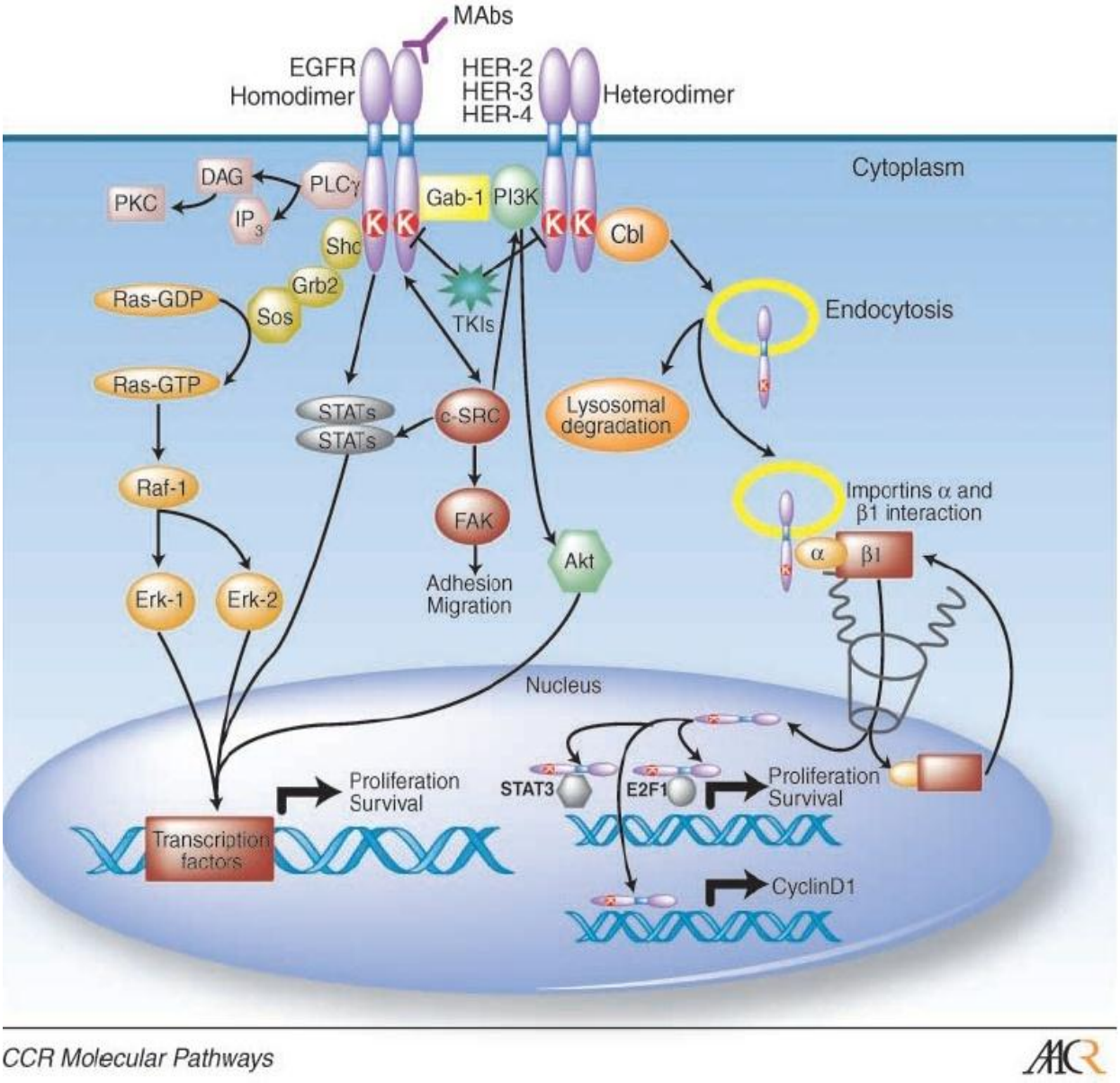

Figure 12. Activation of EGFR and downstream signaling pathways. Abbreviations: DAG: 1,2-diacylglycerol; IP3: inositol 1,3,5-triphosphate; PLCg: phospholipase Cg; Erk-1: extracellular signal-regulated kinase-1: Erk-2, extracellular signal-regulated kinase-2; FAK: focal adhesion kinase; PKC: protein kinase C. Adapted from Scaltriti and Baselga (2006).

Amplification of EGFR gene and increase in transcription or translational processes could be the causes for overexpression of EGFR (Bhargava, et al. 2005). Amplification of EGFR gene was shown in various studies with a range of 8-14 percent among breast cancer cases (Al-Kuraya, et al. 2004; Bhargava, et al. 2005; Quintela, et al. 2005). It was also associated with aggressive tumor phenotype and poor prognosis (Kim, et al. 2001; Quintela, et al. 2005; Tsutsui, et al. 2002a). Hypoxia was found to be another factor for the EGFR overexpression during cancer development and progression. Hypoxia regulates EGFR expression by inducing transcription and translation (Nishi, et al. 2002).

The other oncogenic ability of EGFR is gained from the concurrent overexpression of EGFR and other HER receptors. HER2 lacks ligand binding domain and HER3 has no functional kinase domain. Hence, HER2 and HER3 require heterodimerization for their effective signal transduction pathways. Such concurrent expressions have been reported in various types of cancer including breast (O'Donovan and Crown 2007). Heterodimerization between EGFR and HER2 has been found to be one of the powerful dimer in cellular transformation. Such heterodierization enables the signaling process to be continuous due to reduced endocytosis of HER2. Moreover, EGFR heterodimerization with HER3 enhances cell survival through PI3K/Akt signaling pathways and results in increased resistance against EGFR-targeted treatment (Engelman, et al. 2007; Sergina, et al. 2007; Wang, et al. 1999).

Expression status of EGFR has been suggested as a potential biomarker to be used for clinical management of breast cancer (Faratian and Bartlett 2008). The signaling cross-talk between ER and growth factor receptor pathways lead to resistance against endocrine therapy (Figure 13). Growth factors promote ERα-mediated gene expression by ligand-independent mechanism. In many studies, association of EGFR expression with poor response for tamoxifen treatment have

been reported in breast cancer (Chan, et al. 2006; Morabito, et al. 2003; Schiff, et al. 2004). Anti-EGFR therapies might benefit EGFR overexpressing breast cancer cases and could be used as a predictive marker for EGFR targeted therapies although standardization of its measurement and interpretation is required (Chan, et al. 2006; Faratian and Bartlett 2008).

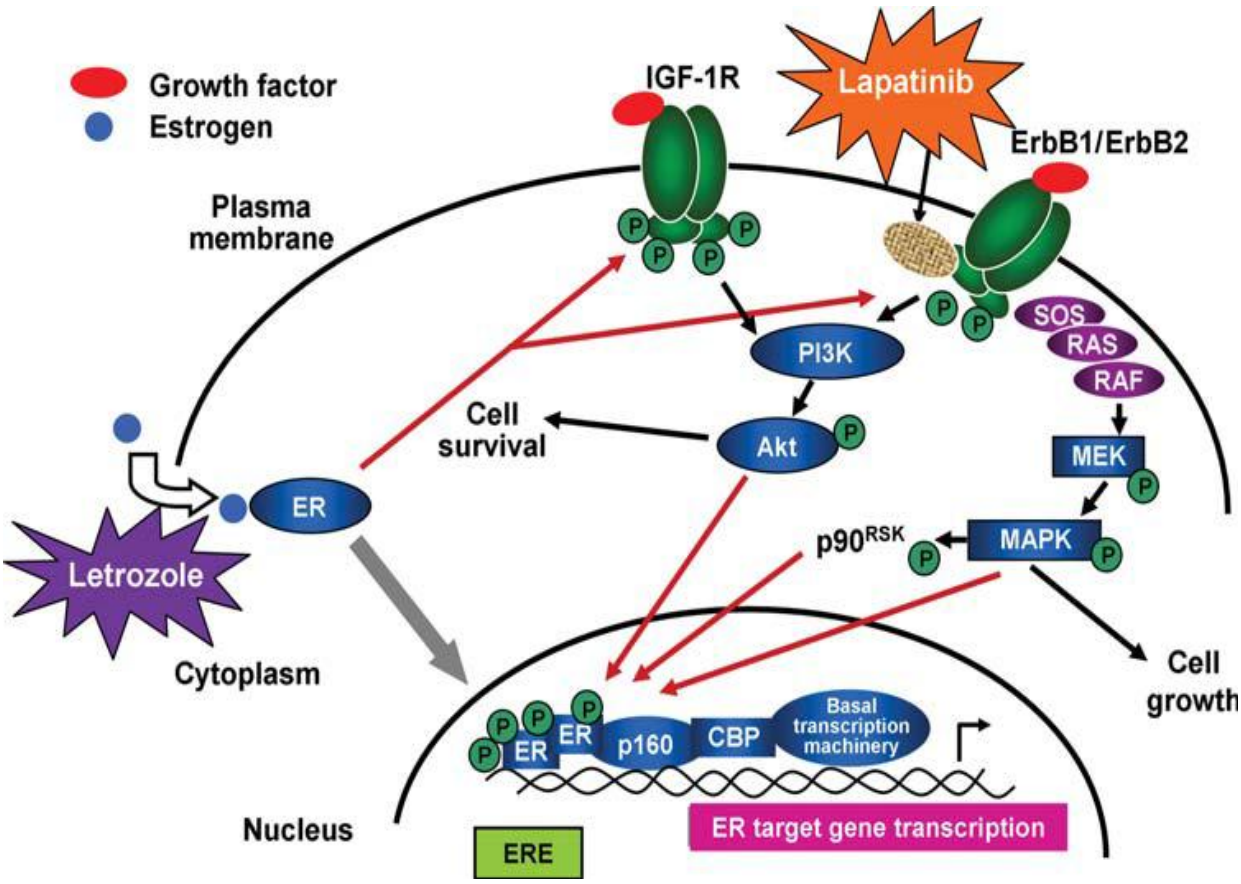

Figure 13: Cellular signaling crosstalk between ER and EGFR (ErbB1)/HER2 (ErbB2) pathways in endocrine-resistant breast cancer. Abbreviations: ER, estrogen receptor; ErbB1, human epidermal growth factor receptor 1; ErbB2, human epidermal growth factor receptor 2; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase kinase; P, phosphate; p90RSK, p90 ribosomal S6 kinase; p160, p160 steroid receptor co-activator protein(s); PI3K, phosphatidylinositol-3-kinase; PTEN, phosphatase and tensin homologue deleted on chromosome 10; RAF, murine leukemia viral oncogene homologue 1; SOS, son-of-sevenless guanine nucleotide exchange factor. Adapted from Vogel et al. (2010).

Angiogenesis is one of the cancer hallmarks that have been shown as a vital process in cancer progression. During tumor angiogenesis, numerous pro-angiogenic factors are involved. Among these, VEGF family members have been recognized to play fundamental roles and also shown to be overexpressed in breast cancer via activation of their receptors, namely, VEGF receptors 1-3 (VEGFR1, VEGFR2 and VEGFR3) (Cimpean, et al. 2008; Dimova, et al. 2014; Racia, et al. 2008).

So far, five members of VEGF family have been known to be expressed in mammalian tissues, namely, VEGF-A, VEGF-B, VEGF-C, VEGF-D and PlGF (Shibuya 2003). VEGF-A is the first type of VEGF identified and recognized as the main blood endothelial cell mitogen. VEGF-B is mainly found in myocardial tissues whereas VEGF-C and VEGF-D have been well known for their role in lymphangiogenesis. Placental growth factor (PIGF) was first identified in the placenta and mainly important for embryonic development (Nagy, et al. 2007; Wirzenius, et al. 2007). These VEGF family members bind selectively to three types of receptors comprising VEGFR-1 (also known as Flt-1) which is usually expressed by blood endothelial cells, VEGFR-2 (also known as KDR/Flk-1) which is predominantly expressed by blood endothelial cells and to some extent by collecting lymphoendothelial cells, and VEGFR-3 (also known as FLT-4) whose expression is limited to lymphatic system and plays a major role in lymphangiogenesis. In physiological condition, expression of VEGFR2 is predominant as compared to VEGFR1 whereas, in pathological condition of angiogenesis, both VEGFR2 and 3 are overexpressed (Nagy, et al. 2007; Niu and Chen 2010; Shibuya, 2006; Takahashi and Shibuya 2005). The VEGF family members can be classified into three categories based on their selectivity for their receptors. VEGF-A can bind to both VEGFR1 and VEGFR2 whereas VEGF-B and PIGF can

bind only to VEGFR1. The other group, VEGF-C and VEGF-D, binds manly to VEGFR-3 but also binds to VEGFR-2. (Clauss 2000; Takahashi and Shibuya 2005) (Figure 14).

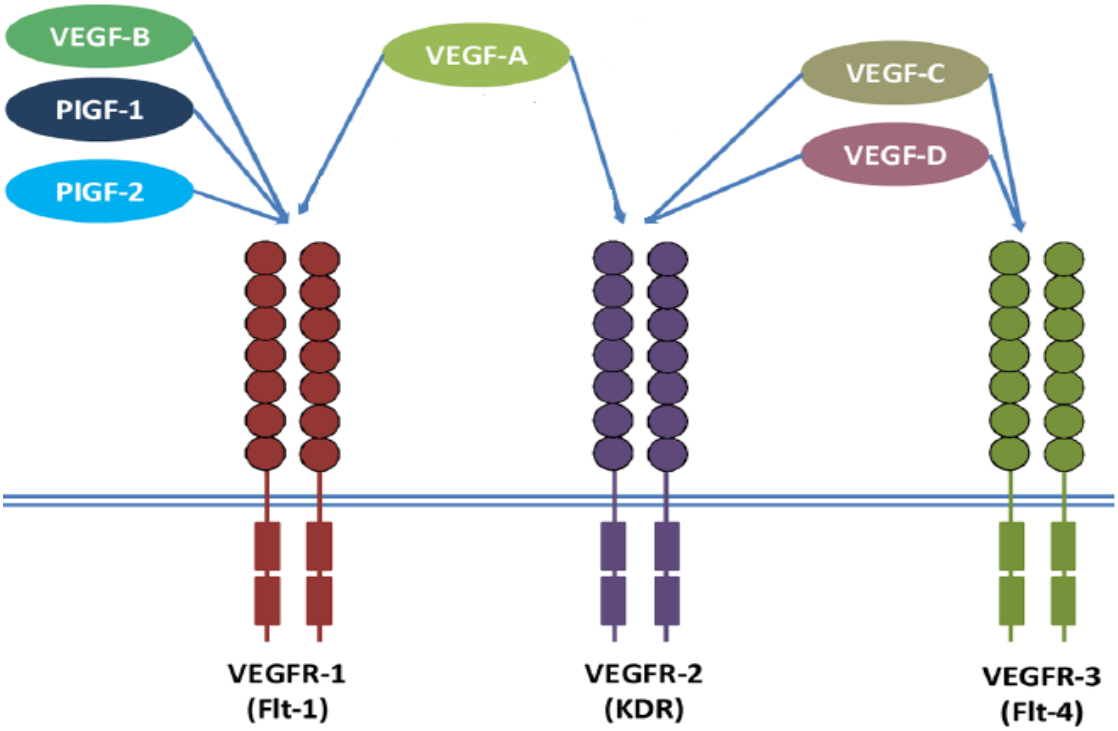

Figure 14: Selective binding of VEGF family members with their receptors. Adapted from Niu and Chen (2010)

VEGF-A was initially discovered by Senger and his collogues and also by Ferrara and his collogues in 1983 and 1989 respectively as vascular permeability factor (VPF) (Ferrara and Henzel 1989; Senger, et al. 1983). Different isoforms of VEGFA have been identified so far and generally classified as proangiogenic isoforms (VEGF-Axxx isoforms) and anti-angiogenic isoforms (VEGFAxxxb). The proangiogenic isoforms are up-regulated whereas the anti-angiogenic isoforms are down-regulated in tumors (Arconde´guy, et al. 2013; Harper and Bates 2008) (Figure 15). Hence, VEGF-A has been identified as the most potent angiogenic factor which iduces endothelial cell gene expression, proliferation, survival and migration during

tumor development (Else, et al. 2011; Fischer, et al. 2008; Li, et al. 2009). These proangiogenic VEGFA isoforms also show variation in terms of solubility and abundance. For instance, VEGF-A121, VEGF-A165 and VEGF-A189 show complete, moderate and very low solubility respectively. VEGF-A121 and VEGF-A165 are the most abundant isoforms (Ferrara 2002).

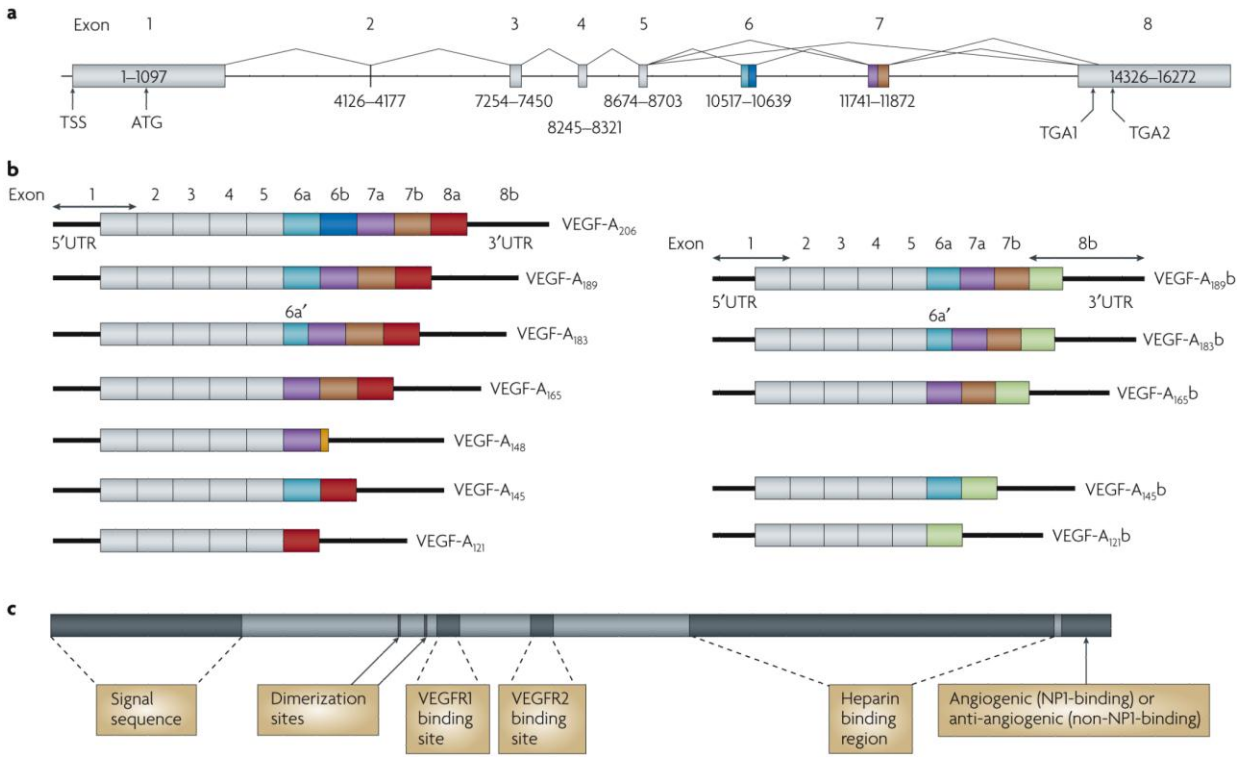

Figure 15: Schematic representations of gene, mRNA and protein structures of human VEGFA. a) VEGFA gene structure. The translation start site (ATG) in Exon 1 and the alternative stop codons (TGA1 and TGA2) at exon 8 are shown. b) The mRNA products of proangiogenic and antiangiogenic VEGFA isoforms. Selected splicing at exons 6, 7 and 8 generate the different isoforms. C) VEGFA protein structure containing different regions. Abbreviations: TSS, transcriptional start site; UTR, untranslated region . Adapted from Harper and Bates (2008)

There are several factors which increase the expression of VEGF-A, among which, hypoxia has been found as the major stimulus for the expression (Blancher, et al. 2001). Apart from hypoxia, there are many other factors which can also stimulate expression of VEGFA including hormones (eg. estrogen, progesterone and thyroid hormones), TGF family, EGF and platelet derived growth factor (PDGF) (Nagy, et al. 2007).

The main VEGFA-induced signaling pathways involve the phospholipase C (PLC)-□/protein kinase C (PKC) cascades that lead to cell proliferation, the PI3K/Akt pathways which result mainly in cell survival, and activation of endothelial nitric oxide synthase (eNOS) to increase vascular permeability. It also activates other downstream pathways such as fokal adhesion kinase pathways which play important role in endothelial cell migration (Grunewald, et al. 2010; Li, et al. 2008) (Figure 16).

Since VEGF A plays a central role in tumor angiogenesis, it was shown to be associated with poor prognosis (Linderholm, et al. 2000a; Morabito, et al. 2003). Shorter overall survival was also significantly correlated with higher expression of VEGF-A in breast cancer cases (Mohammed, et al. 2007). Hence, assessment of VEGF overexpression can categorize the patients who primarily demands VEGF-targeted therapies (Gasparini, 2000; Morabito, et al. 2003).

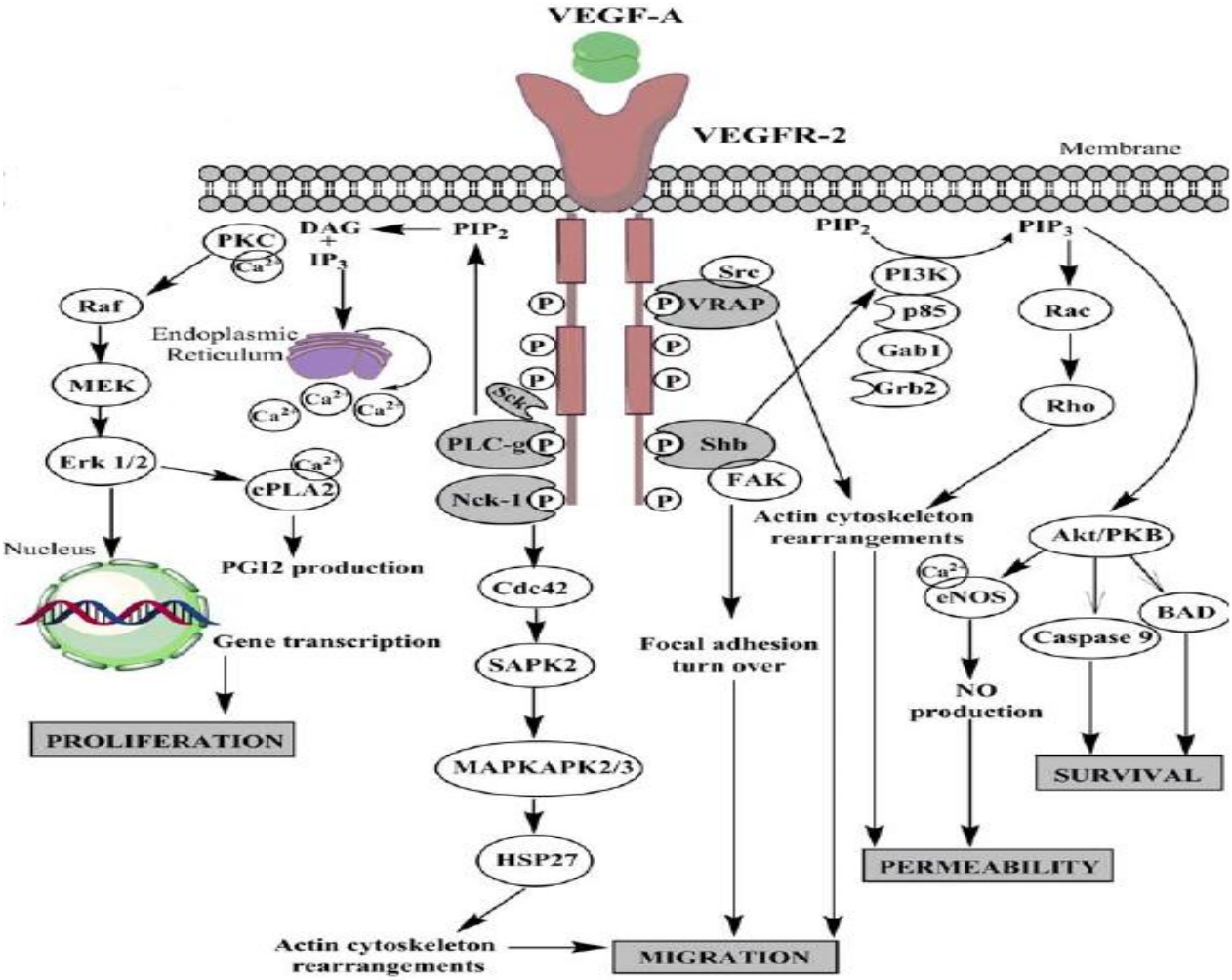

Figure 16. Activation of VEGFR2 by VEGF-A and downstream signaling pathways. Abbreviations: VEGF-A: Vascular Endothelial Growth Factor-A; PLC-g: Phospholipase C-gamma; phospholipase C-gamma; PIP3: Phosphatidylinositol 3,4,5-trisphosphate; DAG: Diacylglycerol; IP3: Inositol Trisphosphate; PKC: Protein kinase C; MEK: MAPK/Erk kinase; Erk 1/2: Extracellular signal-regulated kinase; cPLA2: Cytoplasmic phospholipase A2; Nck-1: Nck adaptor protein; Cdc42: Cell division control protein 42 homolog; SAPK2: Stress-activated protein kinase; MAPKAPK2/3: MAP kinase activated protein kinase-2/3; HSP27: Heat shock protein-27; VRAP: VEGF-receptor associated protein; Src: Rous sarcoma oncogene cellular homolog; Shb: src homology 2 domain containing adaptor protein; FAK: focal adhesion kinase; PIP2: phosphatidylinositol(4,5)bisphosphate; PIP3: phosphatidylinositol(3,4,5)trisphosphate; PI3K: phosphatidylinositol-3 kinase; Gab1: grb2 associated binding protein; Grb2: growth factor bound 2; Rho: rhodopsin; Akt/PKB: Protein kinase B; eNOS: endothelial nitric oxide synthase; Caspace 9: Cysteine Aspartic Acid Specific Protease; BAD: B cell lymphoma 2 (Bcl-2)-associated death promotor; NO: Nitric Oxide. Adapted from Vladimir et al. (2013).

1.3. Significance of the Sudy

The present work could provide additional information to the limited study reports regarding the molecular characteristics of breast tumor in Ethiopian population. Understanding expression status of the standard molecular markers (ER, PR and HER2) could also provide information regarding the benefit of the current treatment strategies.

Although therapeutic selections for breast cancer are mainly based on evaluation of clinicopathologic features and standard molecular markers, treatment responses and overall survival of these patients is still challenging. Hence, further insight in to tumor characteristics through investigation of additional biomarkers is important. Among these, VEGF and EGFR have been reported to be potential diagnostic and predictive biomarkers in breast cancer. This is the first of- its- kind study in Ethiopian population to explore the expression of these potential biomarkers and their association with other clinico-pathological and classical molecular biomarkers. Hence, frequencies of VEGF and EGFR expression as well as association with clinicopathological parameters help further understanding of breast cancer biology.

This study also provides valuable information for researchers to work on the underlying mechanism of breast tumor development and to find the way to improve current treatment strategies. Because of limited resources in our country, this work will guide future efforts in the region to determine whether an emphasis to be given to biological variability or socio-economic factors.

1.4. Hypothesis

Null hypothesis (H_0)

- There is no association among breast cancer IHC biomarkers and histopathological features in breast cancer patients.

Alternative hypothesis (H_1)

- There is a significant association among the IHC biomarkers and histopathological features in breast cancer patients.

CHAPTER TWO

2. OBJECTIVE

2.1. General Objective

- The general objective of this work is to analyze histopathological and IHC biomarkers of breast cancer tissue samples and correlations among them.

2.1.1 Specific Objectives

- To evaluate the incidence of molecular biomarkers (ER, PR, EGFR and VEGF-A) expression in the breast tumor tissues.
- To evaluate the association of ER and PR statuses with histopathological features.
- To correlate EGFR and VEGF-A statuses with histopathological features.
- To examine the association of EGFR and VEGF-A with the ER and PR
- To identify the interrelationships among morphological features and molecular markers

CHAPTER THREE

3. MATERIALS AND METHODS

3.1. Study Area

This study was done in collaboration between Department of biochemistry, Addis Ababa University College of Health Sciences (AAU, CHS) and Department of internal medicine, hematology/oncology unit, University of Michigan, USA. AAU, CHS is a big government institution located in Addis Ababa, the capital city of Ethiopia. The college comprises School of Medicine (SOM), School of Pharmacy (SOP), School of Allied Health Sciences (SAHS), School of Public Health (SPH) and tertiary level teaching hospital (Tikur Anbesa Specialized Hospital, TASH). The college offers health science trainings in eight undergraduate and 70 postgraduate trainings. TASH is the largest specialized referral hospital in the country with more than 700 bed capacity that serves as a center for health professional trainings and several research activities. It also provides in-patient and out-patient clinical services for those reffered to this hospital from other hospitals and health centers. Moreover, it provides clinical and histopathology diagnostic services for most referral and regional hospitals as well as health clinics.

With regard to breast cancer, fine needle aspiration cytology (FNAC) is routinely performed for patients presented with breast lump. Clinically observed and pathologically confirmed breast cancer patients undergo mastectomy followed by radiotherapy, hormonal and chemotherapy. Because of lack of facilities, decision of treatment plan is based on diagnosis of clinical and morphological characteristics with out assessment of the common receptor statuses such as ER, PR and HER2. But compared to other hospitals in the country, it provides better cancer-related

in-patient and outpatient clinical services at the oncology unit. TASH is the only hospital in the country with radiotherapy center.

The University of Michigan medical center in the main campus has eight buildings that comprise Rogel Cancer Center, C.S. Mott Children's Hospital and Frankel Cardiovascular Center. More over, it has 25 health centers in Ann Arbor town and its suroundings. The Rogel Cancer Center has Breast Care Center and Breast Oncology Program where diagnosis and treatment services for breast cancer patients are given through team-based approach after reviewing and deciding treatment plan for individual cancer patient. Although the treatment is unique based on the type of patient, treatment services include surgery, external radiation therapy after breast conserving or mastectomy, endocrine and chemotherapy. Moreover, clinical trials are parts of the breast cancer treatment for needy patients. Breast cancer research program under breast oncology program in the center is one of the most comprehensive program which consists of clinical and laboratory investigators led by Daniel Hayes (M.D.) and Sofia Merajver (M.D., PhD). Several researches are undergoing in relation with screening prevention, and clinical trials of new treatment strategies for patients with existing breast cancer.

3.2. Study Design

A retrospective evaluation of morphological and immunohistochemical features was done among invasive breast cancer patients who underwent modified radical mastectomy (MRM) at two referral hospitals, namely, St. Paul Millennium Medical College (SPMMC) and Minilik II Memorial Referral Hospital (MIIMRH). Selection of the cases was based on clinical diagnosis (by clinical oncologist) and confirmed by pathologic databases. Formalin-fixed paraffin-embedded (FFPE) tissue samples from all the cases were used to evaluate the expression statuses of molecular markers and their correlation with histopathological parameters among the

breast cancer patients who underwent MRM. The histopathological data of each study subject were obtained from pathology reports and the expression statuses of the molecular biomarkers were detected by IHC analysis.

3.3. Study Population

The present study population involved confirmed female invasive breast carcinoma cases who underwent MRM involving axillary lymph node dissection. The surgery was performed from the period of June 2014 to June 2015 at SPMMC and MIIMRH. The resected tissues were sent to two referral hospitals (SPMMC and TASH) for morphological analysis and FFPE preparation. Among these FFPE blocks, some of them were excluded from the study because of different reasons such as being undetectable or non-invasive nature of the tumors.

3.4. Study participants

In the present study, the breast cancer cases were selected based on the following inclusion and exclusion crieteria. Hence a total of 85 appropriate FFPE blocks were included in our study for IHC analysis. Subjects were coded first instead of name for analysis.

3.4.1. Inclusion Criteria

- Adult females with age > 18 years at the time of diagnosis.
- Clinically and histologically confirmed breast cancer cases
- Invasive breast cancer cases
- Cases with MRM and having available FFPE blocks

3.4.2. Exclusion Criteria

- Male breast cancer cases
- Females aged < 18 years of age
- Non-invasive breast cancer cases
- Breast cancer cases diagnosed only with FNAC

3.5. Sample Size

According to the hospital-based data which was obtained from five years records (2011-2015) by surgery departments of the two hospitals where the breast mastectomies were done, an average of 120 females underwent MRM annually. The prevalencence of invasive female breast cancer in Ethiopian population is not yet studied. Hence, the sample size was calculated using the formula for finite population as described by Naing, et al. (2006). Hence, the sample size calculation was done as follows.

$$\mathbf{n' = \frac{NZ^2 P(1-P)}{d^2 (N-1) + Z^2 P(1-P)}}$$

Where, n' = sample size with finite population correction

N = size of the target population = 120

Z = Z statistic for 95% level of confidence = 1.96

P = Estimated proportion of breast cancer patients with hormone receptors in Ethiopian population = 65% (48.75) (Kantelhardt, et al. 2014_a).

d = margin of error = 5%.

Hence, the minimum sample size (n) calculated was 90.

3.6. Collection of Tissue Samples, Processing, Staining and Histopathologic Data

Table 6. Major Chemicals and Equipments

Chemicals	Manufacturer	Chemicals	Manufacturer
10% formalin	(Sigma-Aldrich, Netherland)	Citric Acid	Sigma Aldrich, Germany
Paraffin	(Alexandria, Egypt)	Aluminium Potassium Sulfate	Fine Chemicals, India
Xylene	(Lab Chemie, India)	Sodium Iodate	Fine Chemicals, India
Graded ethanol (Absolute, 95%, 85%, 70%, 50%)	(Nice Chemicals, India)	Chloral Hydrate (AR)	Uni-Kem, USA
Hematoxylin Powder	(FLUKA Chemie, Swizerland)	Equipments	Manufacturer
Eosin	(BDH Chemicals, UK)	Oven	Thermo Scientific, North America
DPX mountant	(Labtech, Australia)	Autostainer Link	(Dako, North America)
FLEX TRS High pH Retrieval buffer (pH 9) (50x)	(Dako, North America)	Dako PT Link	(Dako, North America)
Proteinase K RTU	(Dako, North America)	Microtome	SLEE, Germany
EnVision™ FLEX Peroxidase-Blocking solution (RTU)	(Dako, North America)	Water bath	SLEE, Germany
EnVision™ FLEX Wash Buffer (20x)	(Dako, North America)	Microscope	Olympus,Japan
EnVision™ FLEX+ Mouse/Rabbit (LINKER)	(Dako, North America)	FLEX IHC Microscope Slides	(Dako, North America)
EnVision™ FLEX /HRP (RTU)	(Dako, North America)		
EnVision™ FLEX Substrate Buffer	(Dako, North America)		
EnVision™ FLEX DAB+ Chromogen	(Dako, North America)		

3.6.1. Collection of Tissue Samples

The routine procedure for collection, transport and processing of surgically resected samples for histopathological analysis is briefly described as follows. After surgery, the whole breast tissue and axillary lymph nodes specimens were dipped in container containing 10% formalin and transported to department of pathology for further processing, FFPE preparation and morphological analysis. Those tumors obtained from MIIMRH are sent to AAU, CHS whereas, tissues removed at SPMMC were processed at the same place at pathology department by routine pathology procedures.

3.6.2. Tissue processing

After arrival of the gross breast tissues to the pathology unit, they were sliced into pieces 4-5 cm and immersed in 10% formalin again. Then the tissues were trimmed into 3-4 mm and put in tissue cassette to be processed. Under Tissue Tek VIP Processor, the tissues were fixed, dehydrated with graded ethanol (50%, 70%, 85% and absolute ethanol) and impregnated with paraffin wax.

3.6.3. Hematoxylin and Eosin (H & E) Staining

The H & E staining as part of a routine procedure was done at AAU, CHS and SPMMC. The FFPE tissues were first sectioned at 4µm by microtome, placed on microscope slide, dehydrated, deparaffinized and rehydrated using graded ethanol and water. The slides were then stained with Mayer's Hematoxylin followed by washing with water and stainied with eosin. Finally the tissues were dehydrated by dipping in graded ethanol and xylene. Finally the slides were mounted with DPX**,** air-dried and observed under microscope**.** Classification of the breast carcinoma samples into different histologic types was done according to the WHO criteria as

described previously. Histological grading of the tumors was also done according to the method of Scarff-Bloom-Richardson modified by Elston-Ellis, with the principles recommended by pathologists from Nottingham City Hospital as described in the previous section.

3.6.4. Histopathological Data

Due to inadequacy of clinical information on patients' cards for significant number of the breast cancer cases, we collected the patients' information from histopathology requesting and reporting form that have been prepared by the pathology department. The age of patients during surgery and laterality of the breast cancer were also registered from the pathology reporting form.

3.7. Immunohistochemical Analysis

All IHC analyses were performed at University of Michigan, USA. A total of 85 invasive breast cancer FFPE samples from individual cases were selected for IHC staining. For preparation of IHC slides, the paraffin blocks were cut at 5μm thickness section followed by deparaffinization using xylene and graded alcohol. The slides were rehydrated by distilled water. Heat induced retrieval method was done with FLEX target retrieval solution (TRS), High pH (9). The next procedures were performed by using Dako Autostainer Link (Figure 17) to be certain for similar staining situation in every run.

Figure 17. Dako Autostainer Link 48

The principle of the procedures is briefly described as follows. After endogenous peroxidase blocking, the primary antibodies for ER, PR and VEGF (Table 7) were applied and washed with wash buffer. EnVision FLEX LINKER (rabbit) was applied and washed. The slides were then immersed in EnVision™ FLEX /HRP detection reagent (Agilent, Dako) consisting HRP labeled polymer and multiple goat secondary antibodies. Finally, EnVision™ FLEX DAB+ Chromogen (Agilent, Dako) was applied to the slides and washed with distilled water. Counterstaining was done with Harris hematoxylin followed by dehydration with graded ethanol and xylene. In the case of EGFR, epitope retrieval was performed with enzymatic pre-treatment using Proteinase K ready-to-use reagent. The FLEX+ Mouse EnVision System was used for detection. The IHC staining steps was schematically shown in figure 18.

Table 7. Sources and dilutions of primary antibodies

Clone	Type of Antibody	Manufacturer	Dilution
SP1 (Anti-ER)	Rabbit monoclonal	Cell Marque, Rocklin, CA	1: 50
Y85 (Anti-PR)	Rabbit monoclonal	Cell Marque, Rocklin, CA	1: 40
31G7 (Anti-EGFR)	Mouse monoclonal	Invitrogen, Camarillo, CA	1: 50
A-20 SC-152 (Anti-VEGF)	Rabbit polyclonal	Santa Cruz, CA	1:250

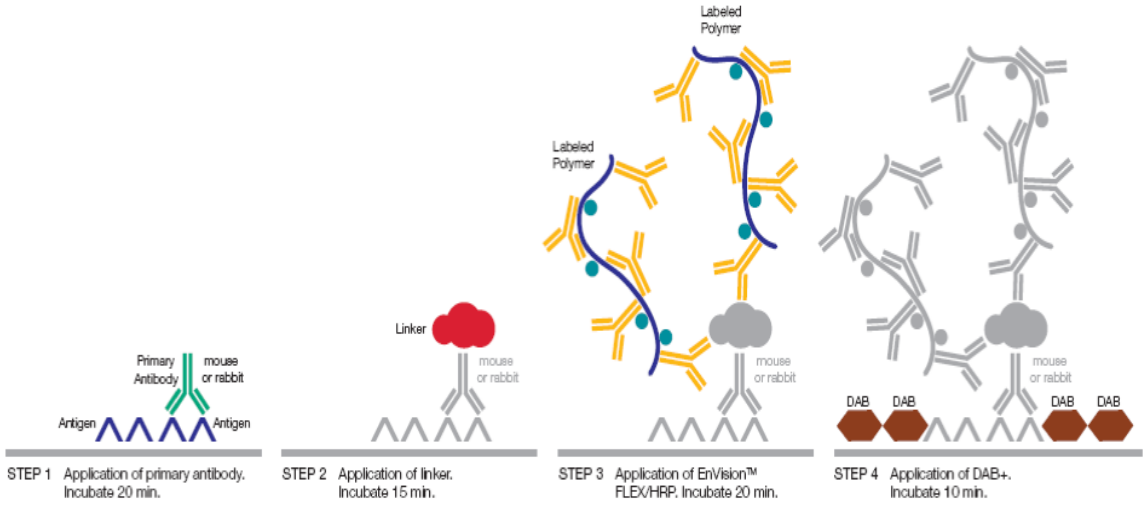

Figure 18. Schematic representation of IHC Steps by FLEX+ EnVision Detection System

The IHC slides were scanned using Aperio slide scanning system (Aperio® Technologies, Inc., Vista, CA) (Figure 19) which captures high resolution, whole slide images of microscopic specimens. Included in this system are algorithms for nuclear, cytoplasmic, and membrane labeling quantification. This digital image analysis is a standardized, reliable method to grade specimens with sufficient labeling quality in an efficient manner.

Figure 19. Aperio digital slide scanner

With every round of immunostaining, tumors which are known for expression of antigen of interest were included as positive controls and primary antibodies were omitted for negative controls in each set of the staining slides (Figure 20).

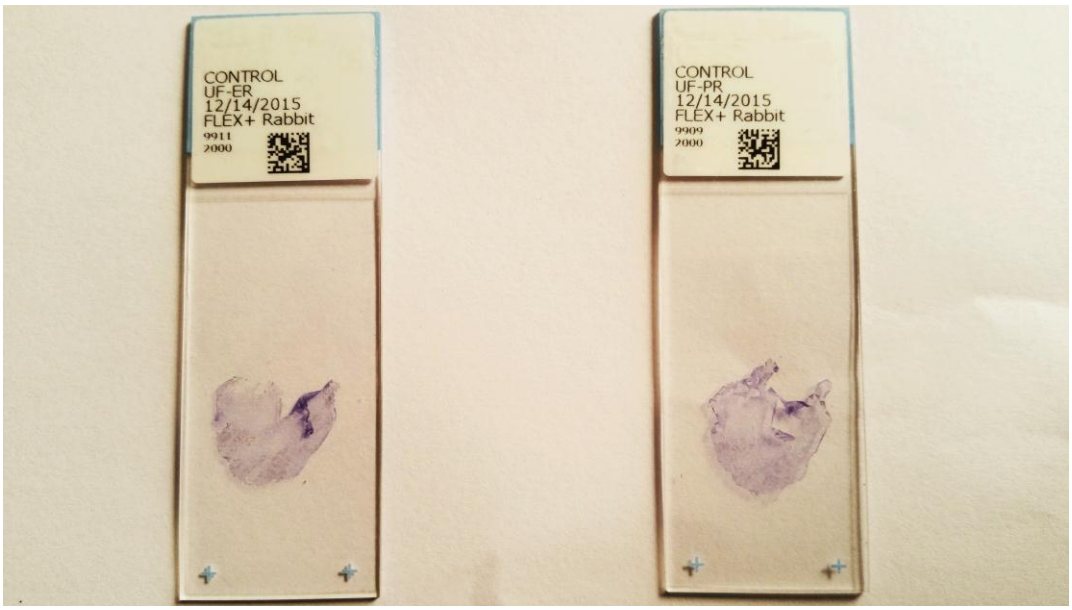

Figure 20. Positive control slides for ER and PR

Evaluation of the IHC stained slides of hormonal receptors was made on the basis of Allred's scoring system as stated in Fitzgibbons, et al. (2014). According to this system, proportion and

intensity scores are combined in calculating the total score (Table 8). The total score was calculated by summation of the two scores with the range of 0-8. The total score of 0-2 were considered as negative whereas 3-8 were considered as negative.

Table 8. Allred Score for ER and PR Grading

% Positive Cells	Proportion Score	Intensity	Intensity Score
0	0	None	0
Less than 1	1	Weak	1
1 to 10	2	Intermediate	2
11 to 33	3	Strong	3
34 to 66	4		
> 67	5		

EGFR protein expression score was reported based on previous publications (Kim, et al. 2006; Mokhtari, et al. 2012). EGFR staining was considered positive when at least 10% of tumor cells showed strong membranous staining. Therefore, tumors with fewer than 10% of cells were considered as negative. Normal adjacent or entrapped glands were not factored into the estimated grade. Stromal labeling was not considered as positive staining.

Scoring of VEGF-A cytoplasmic expression was characterized by semi-quantitative method as described by Dhakal, et al. (2012). The labeling of intensity and proportion scores are shown in table 9. The combined score of VEGF-A expression was calculated by multiplying the proportion and intensity scores with a range of 0-9. Scores from 0-3 were categorized as no/ low, 4-6 as moderate and greater than 6 were categorized as high expression. In the current study, tumors having moderate or strong VEGF-A expression were categorized as positive while the rest were considered as negative during statistical analysis.

Table 9– Scoring system for VEGF-A protein expression

Proportion	Proportion Score	Intensity	Intensity Score
0	0	No staining of tumor cells	0
<10% of positive cells	1	Weak staining	1
10-50% of positive tumor cells	2	Moderate staining	2
>50% of positive tumor cells	3	Strong staining	3

3.8. Statistical Analysis

Statistical analysis of our data was done using the SPSS 24.0 software (SPSS Inc., Chicago, IL, USA). Descriptive statistics was used to summarize the data regarding the histopathologic characteristics and molecular marker expressions. The Pearson's chi-square test was used to determine the associations between categorical variables. Whenever necessary, Fischer's exact test was applied if the expected values for a two by two contingency table were less than five. Two-tailed P value was set < 0.05 to consider the result to be statistically significant.

3.9. Ethical Considerations

The ethical approval for our study was obtained from department ethical review committee (DERC), Institutional Review Board (IRB) of CHS, AAU, (Meeting No.: 059/14; Protocol Number: 010/14/Bioch.; and Meeting No: 007/2015; Protocol number: 010/14/Bioch.) (See annex IA & IB) and the Federal Health Research Ethics Review Committee, Ministry of Science and Technology, Ethiopia (Ref. No. 310/023/2015) (See annex II). Material transfer agreement was signed between Breast and Ovarian Cancer Risk and Evaluation Program, Department of Internal Medicine, University of Michigan and Department of Biochemistry, College of Health Sciences, Addis Ababa University (See annex III).

CHAPTER FOUR

4. RESULTS

4.1. Age Distribution and Histopathological Characteristics

In this study, breast cancer patients who were diagnosed and underwent the MRM between June 2014 and June 2015 at two government hospitals were selected. The age distribution of the patients ranged from 22 to 75 years (mean ± standard deviation (SD), 42.14±11.96). The frequency percentages of the age groups were shown in figure 21. Regarding the laterality of breast cancer cases, it was also found that 40% (34/85) of the patients had tumor on the right breast, and 44.7% (38/85) had on the left breast. While, the remaining 15.3% study participants do not have registered laterality on their medical file.

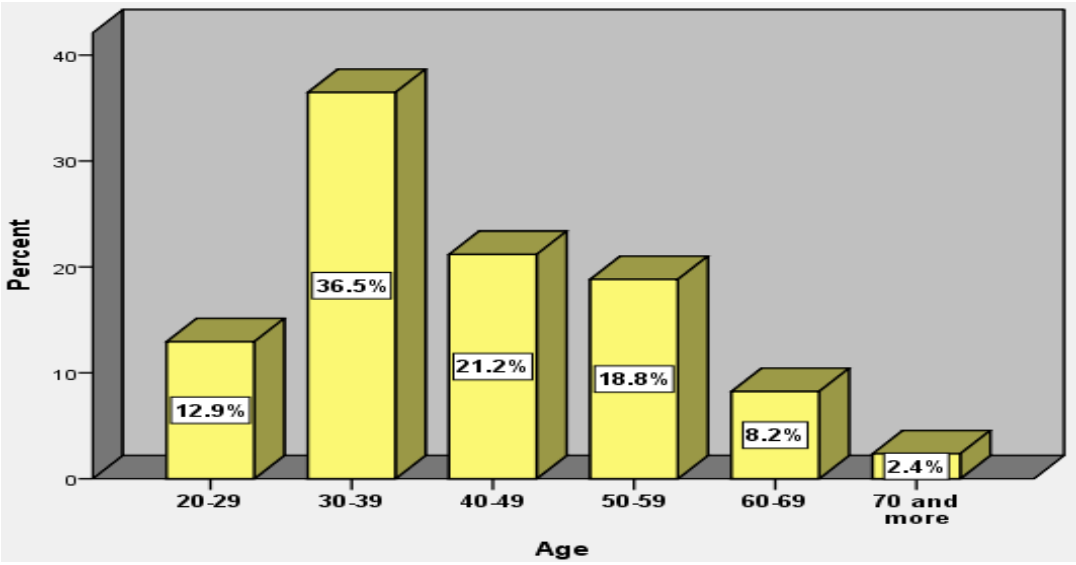

Figure 21. Frequency percentage distribution of breast cancer cases by age at diagnosis

In our study, 89% (74/83) of primary breast tumor samples were identified as IDC type. Other types of morphologies include ILC, invasive ductal and lobular, and other invasive types (two mucinous and one papillary) of carcinomas as summarized in table 10. More than 60% (49/80) of the tumor samples belong to T3 and T4. Similarly, more than 70% (57/77) of the breast

cancer cases showed axillary lymph node metastasis. Among the histologically assessed tumor grades, 51% (36/71) were poorly differentiated tumors (G3), 38% (27/71) were moderately differentiated (G2) and the remaining 11.3% were well differentiated (G1) tumors. The lymph node status of 14 cases in our study samples was not assessed.

Table 10. Distribution of histopathological characteristics of the breast tumor samples

Histopathological Features	Count	Percentage frequency (%)
Histological subtype		
Invasive Ductal Carcinoma	74	89.2
Invasive Lobular Carcinoma	3	3.6
Mixed ductal and Lobular Carcinoma	3	3.6
Other Invasive Types	3	3.6
Missing	2	
Tumor Size		
T1	9	11.3
T2	22	27.5
T3	26	32.5
T4	23	28.8
Missing	5	
Histologic Grade		
G1	8	11.3
G2	27	38.0
G3	36	50.7
Missing	14	
Lymph Node Status		
N0	20	26.0
N1	30	39.0
N2	23	29.9
N3	4	5.2
Missing	8	

4.2. Expression Statuses of the IHC Markers

The protein expressions were determined by IHC techniques. Majority of the study samples were positive for ER (62/84) and PR (50/83) expressions, EGFR positivity was observed only in 21.7% (18/83) of the cases. With regard to VEGF-A protein expression, majority (53/83) of patient tumors expressed it at low levels whereas only few (11/83) of them were negative for VEGF-A expression (**Figure 22**). The frequency distribution of the IHC markers' expression statuses are shown in table 11.

4.3. Inter-associations of ER, PR, EGFR and VEGF-A

A total of 83 (97.6%) cases were analyzed for the correlation between expressions of ER and PR in the breast cancer cases. Majority (49/62, 79.0%) of the ER positive breast cancer tissues also expressed PR. Whereas, most of the ER negative tissues did not express PR (20/21, 95.2%). Statistically, very significant association was observed between the expressions of ER and PR in breast cancer tissues ($P < 0.005$) (Table 12).

Table 11: Frequency distribution and percentage for the status immunohistochemical biomarkers

Variables	Status	Frequency	Frequency Percent
ER	Positive	62	73.8
	Negative	22	26.2
	Missing	1	
PR	Positive	50	60.2
	Negtive	33	39.8
	Missing	2	
EGFR	Positive	18	21.7
	Negative	65	78.3
	Missing	2	
VEGF-A	High	2	2.4
	Moderate	17	20.5
	Low	53	63.9
	Negative	11	13.3
	Missing	2	

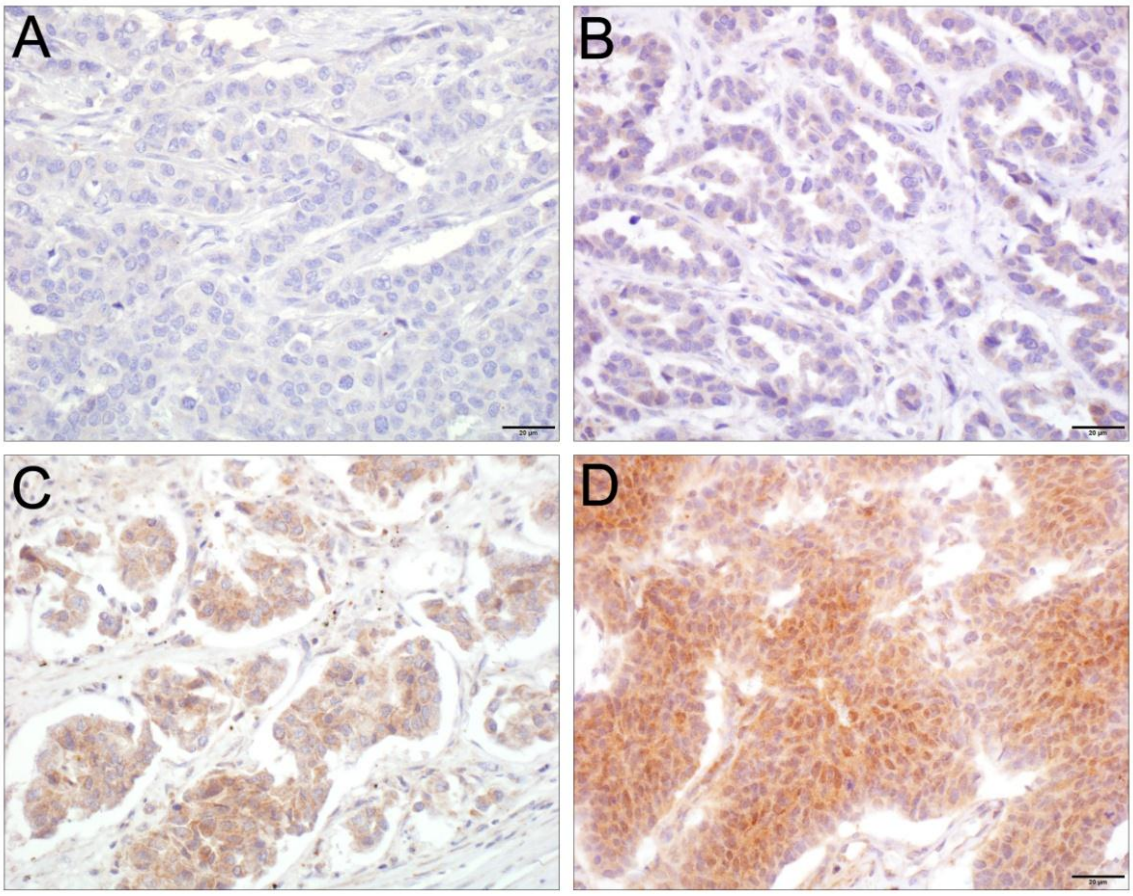

Figure 22. Representative figures for VEGF-A IHC labeling. VEGF-A was labeled as 0 (no staining, A, 40X), 1 (weak staining, B, 40X), 2 (moderate staining, C, 40X) and 3 (strong staining, D, 40X). Bar = 20um.

The association between cellular expressions of ER and the EGFR expression in breast tumor samples was statistically analyzed. The analysis confirmed that significantly inverse association was found between ER status and EGFR expression ($p = 0.01$). Only 14.5% (9/62) of ER positive tumors also co-express EGFR whereas 42.9% (9/21) of ER negative cases were positive for EGFR expression. In comparison of EGFR positivity in PR negative and positive breast tumor tissues, the positivity of EGFR is more in PR negative patients as compared to PR positive breast cancer tissues (33.4% Vs 14%). Like ER, statistical analysis also showed significant inverse relationship between EGFR expression and PR status ($p=0.04$).

On the other hand, non-significant association between expressions of ER and VEGF-A proteins were observed (*P*=0.28). However, 14.3% (3/21) of ER negative cases were positive for strong/moderate VEGF-A expression and 25.8% of ER positive tumors were VEGF-A positive. No significant relationship between PR and VEGF-A was also observed (*P*=0.75).

Table 12. Associations among expressions of ER, PR, EGFR and VEGF-A biomarkers

Guides	PR			EGFR			VEGF-A		
	Positive, N (%)	**Negative, N (%)**	p-Value	**Positive, N (%)**	**Negative, N (%)**	p-Value	**Positive, N (%)**	**Negative, N (%)**	p-Value
ER **Positive** **Negative**	49 (79.0) 1 (4.8)	13 (21.0) 20 (95.2)	< 0.005	9 (14.5) 9 (42.9)	53 (85.5) 12 (57.1)	0.01	16 (25.8) 3 (14.3)	46 (74.2) 18 (85.7)	0.28
PR **Positive** **Negative**				7 (14.0) 11 (33.3)	43 (86.0) 22 (66.7)	0.04	11 (22.0) 8 (25.0)	39 (78.0) 24 (75.0)	0.75

4.4. Associations of Age with Expressions of the IHC Markers

With regard to the association between age of the breast cancers cases and ER expression of the breast tumor, no correlation was observed in our study (p=0.17). However, the ER positivity among the older age groups was a little bit greater than among the younger age groups (84.0% Vs 69.5%). With regard to the association between age group and PR protein expression status, PR negativity was more frequent in younger age group as compared to the older age groups (46.6% Vs 24.0%). Majority of older age breast cancer cases at the time of diagnosis showed positivity in PR expression than younger age group (76% vs 53.4%). Statistical analysis showed

that there was borderline association between expression of PR and age of breast cancer cases (p= 0.05).

Correlation analysis did not show significant difference between the two age groups with regard to EGFR expression status by the breast tumor tissues (p=0.41). However, EGFR positivity in younger women was higher than in older age groups (24.1% vs 16.0%). Statistical approach was also confirmed non-significant association between the status of VEGF-A protein expression and the age of breast cancer cases (p=0.19). However, positivity of VEGF-A is more in older age groups as compared to younger ones (32.0% Vs 19.0%). Association of age groups of breast cancer cases with expression of molecular biomarkers by tumor tissues are shown in in table 13.

Table 13. Relationship between age and IHC biomarkers

Biomarker	**Age <50, N (%)**	**Age >50, N (%)**	**p-value**
ER			
Positive	41 (69.5)	21 (84.0)	0.17
Negative	18 (29.5)	4 (16.0)	
PR			
Positive	31 (53.4)	19 (76.0)	0.05
Negative	27 (46.6)	6 (24.0)	
EGFR			
Positive	14 (24.1)	4 (16.0)	0.41
Negative	44 (75.9)	21 (84.0)	
VEGF-A			
Positive	11 (19.0)	8 (32.0)	0.19
Negative	47 (81.0)	17 (68.0)	

4.5. Association of Age and Histopathological Features

The age of the study participants were correlated with the tumor size. With respect to lower and higher age groups of breast cancer cases, 56 (69.6%) of the younger age breast cancer cases (Age <50 yrs) had larger tumor size (>5 cm). Conversely, majority of the older age groups (Age >50 yrs) have smaller tumor size (< 5 cm, 58.3%). Statistically, a significant inverse association between tumor size and age of the breast cancer cases was observed (p=0.02).

Analysis of the correlation between age and nodal metastasis showed that around 40% of the younger age groups showed higher lymph node involvement (pN2/pN3) while only 25% of the older age groups belong to higher nodal involvement. But no association has been shown between the two parameters (p=0.21). No significant association was also observed between histological grade of tumor tissues and the age groups of breast cancer cases (*P*=0.31). The table below (Table 14) shows the association between histopathologic features and age groups at diagnosis of breast cancer cases.

Table 14. Association of age groups of breast cancer cases with histopathological features

Histopathologic features	Count	Age <50, N (%)	Age >50, N (%)	p-value
Tumor size				
pT1/pT2	31	17 (30.4)	14 (58.3)	0.02
pT3/pT4	49	39 (69.6)	10 (41.7)	
Lymph node status				
pN0-pN1	50	32 (60.4)	18 (75.0)	0.21
pN2-pN3	27	21 (39.6)	6 (25.0)	
Histological grade				
1/2	35	25 (50.0)	10 (47.6)	0.31
3	36	25 (50.0)	11 (52.4)	

4.6. Association of ER and PR Expressions with Histopathologic Features

The ER expression status of tumor tissues showed non-significant association with the size of the breast tumor (p= 0.90). Similar observation was recorded for PR expression status and size of the breast tumor tissues (p=0.88).

With regard to lymph node status, statistical methods showed no significant association between presence of metastatic lymph nodes and ER status in breast tumors. In both higher and no/low lymph node metastasis, similar frequency of ER positivity was observed. Similarly, no statistically significant relationship was observed between the status of lymph node metastasis and expression of PR (*P*=0.62) in breast tumor.

The grades of breast cancer tissues and expression status of ER did not reveal any significant correlation (P=0.11). Though, the rate of ER negativity was higher among G1/G2 tumor tissues as compared to higher grade (grade 3) tumors. In relation with PR expression, the statistical analysis also revealed non-significant correlation (P=0.55) with tumor grade. Similar to ER, PR negativity was more frequent among lower histologic grade (G1/G2) as compared to high grade tumors (47.1% vs. 40.0%). The relationships between histopathologic characteristics and expressions of ER, PR in breast cancer tissues are shown below (Table 15).

Table 15. Associations of ER and PR with histopathological features

Morphological features	ER			PR		
	Positive, N (%)	Negative, N (%)	p-Value	Positive, N (%)	Negative, N (%)	p-Value
Tumor size						0.88
pT1-pT2	23 (74.19)	8 (25.81)	0.9	18 (60.0)	12 (37.5)	
pT3-pT4	35 (72.9)	13 (27.1)		28 (58.3)	20 (62.5)	
Lymph node status						0.62
pN0-pN1	36 (73.5)	13 (26.5)	0.95	28 (57.1)	21 (42.9)	
pN2-pN3	20 (74.1)	7 (25.9)		17 (63.0)	10 (377.0)	
Histological grade			0.11			0.55
G1/G2	22 (62.9)	13 (37.1)		18 (52.9)	16 (47.1)	
G3	28 (80.0)	7 (20.0)		21 (60.0)	14 (40.0)	

4.7. Association of EGFR and VEGF-A Expressions with Histopathologic Features

Regarding the analysis of breast cancer size and EGFR status, there was no statistically significant association between EGFR positivity and tumor size (p=0.61). However, 25.0% (12/48) of larger size tumors were EGFR positive compared to 20.0% (6/30) small size breast tumors. Statistical analysis also did not show significant association of regional lymph node metastasis with EGFR expression status (*P*=0.55). Similarly, no statistically significant association was oserved between the tumor grades and EGFR expression (*P*=0.16) in breast tumor tissues. However, EGFR positivity was more frequent among high grade breast tumors compared with low/intermediate grade tumors (28.6% vs. 14.7%).

Statistical analysis did not show significant association between breast tumor size and VEGF-A over expression (p=0.77). Similar to EGFR, greater frequency of VEGF-positivity was observed among larger size tumors as compared to smaller size tumors (25.5% vs 22.6%). There was also non-significant correlation between the status of lymph node metastasis and VEGF-A expression (*P*=0.52). However, the proportion of VEGF-A positive tumors among tumors with higher lymph node metastasis was greater as compared to tumors with no/low lymph node metastasis. Also, we did not find any significant association of VEGF-A expression with histologic grade of the tumors (*P*=0.82). Comparison of histopathological characteristics with expressions of the potential prognostic factors EGFR and VEGF-A are shown in the following table **(**Table 16).

Table 16. Comparison of histoathological features with expressions of EGFR and VEGF-A

Histopathologic features	EGFR			VEGF		
	Positive, N (%)	Negative, N (%)	p-value	Positive, N (%)	Negative, N (%)	p-value
Tumor size						
pT1-pT2	6 (20.0)	24 (80.0)	0.61	7 (22.6)	24 (77.4)	0.77
pT3-pT4	12 (25.0)	36 (75.0)		12 (25.5)	35 (74.5)	
Lymph node status						
pN0-pN1	12 (24.5)	37 (75.5)	0.55	10 (20.4)	39 (79.6)	0.52
pN2-pN3	5 (18.5)	22 (81.5)		7 (26.9)	19 (73.1)	
Histological grade						
G1/G2	5 (14.7)	29 (85.3)	0.16	8 (22.9)	27 (77.1)	0.82
G3	10 (28.6)	25 (71.4)		7 (20.6)	27 (79.4)	

4.8. Interrelationships between Histopathological Characteristics

Altogether, a total of 74 (87.1%) were examined for the association of regional lymph node status with tumor size. Majority (26/29, 89.7%) of the tumors with smaller size in diameter (pT1/pT2) showed no/few regional lymph node metastasis. The incidence of pN2/pN3 was higher in larger size tumors compared to small sized tumors (48.9% vs. 10.3%). The statistical analysis approved that there was a very significant association between regional lymph node metastasis and size of the breast tumor samples (p=0.001)

With regard to the tumor grades, G3 tumor tissues were predominated in larger tumors compared to G1/G2 tumors (77.1% Vs 54.6%). Whereas, similar distribution of tumor

size/stage were shown in G1/G2 tumors. However, the correlation between the two morphological factors did not reach statistical significance (P=0.07).

No association between tumor grade and lymph node metastases was also observed in this study (p= 0.80). Although majority of both G1/G2 and G3 groups belong to no/lower nodal metastasis, higher nodal involvement (pN2/pN3) was observed in G3 than G1/G2 tumors (39.4% Vs 36.4%). The relationship between the histopathologic features was summarized in table 17.

Table 17. Inter-associations among selected histopathological characteristics

Histopathologic features	Tumor size			Lymph node status		
	pT1-pT2	**pT3-pT4**	**p-Value**	**pN0/pN1**	**pN2/pN3**	**p-Value**
Tumor size						
pT1/pT2				26 (89.7)	3 (10.3%)	0.001
pT3/pT4				23 (51.1)	22 (48.9%)	
Histological grade						
G1/G2	15 (45.4%)	18 (54.6%)	0.07	21 (63.6%)	12 (36.4%)	0.80
G3	8 (22.9%)	27 (77.1%)		20 (60.6%)	13 (39.4%)	

CHAPTER FIVE

5. DISCUSSION

Breast cancer is ranked as the second in prevalence and the fifth cause of death from all types of cancer worldwide. While lung cancer is the most commonly diagnosed cancer type and cancer deaths in males, breast cancer is the most common type of malignancy in terms of incidence and mortality in females (Bray, et al. 2018). Although limited data are available regarding the incidence and mortality of cancer in Ethiopia, breast cancer was identified as the most commonly diagnosed types of cancer among females both in Addis Ababa (the capital city) and selected regional health centers. It was shown also to be the commonest cause of cancer death among females (Memirie, et al. 2018).

Breast cancer is a heterogenous type of disease in terms of its clinical, histopathological and molecular features. Ethnic differences have been shown to be one of the various factors which influence the clinical, histopathologic and molecular features of female breast cancer and their clinical outcomes (Carey, et al., 2006; Chlebowski, et al. 2005). Assessment of clinical, histolpathological and molecular characteristics of breast cancer is crucial in diagnosis, predicting clinical progression of the disease and selection of appropriate therapy (Diaz, et al. 2005; Goldhirsch, et al. 2011). So far, few studies are available regarding the molecular and clinicopathological characteristics of female breast cancer in Ethiopian population. Hence, our present work studied the frequency distributions for expressions of some molecular biomarkers by IHC method and histopathological features as well as the inter-associations between these features among the invasive breast cancer cases in Ethiopia.

Age Distribution and Histopathological Features

In the current study, the mean age of the study participants at diagnosis was 42.1 (age range, 22-75 years). Similar finding was reported by Jiagge, et al. (2016) among Ethiopian breast cancer cases (age range, 23-76 years). But, another study by Shenkutie, et al. (2017) reported the mean age of 46.7 years in Ethiopian populaion with the age range of 22-100 years. The slight difference in the mean age with the current study might be due to the difference in the age distribution of the study participants. Similar mean ages at diagnosis were also reported from other Sub-Saharan countries including Nigeria (46.85) (Anyanwu 2008), Ghana (49) (Jiagge, et al. 2016), Kenya (48) and Democratic Republic of Congo (47) (Bhikoo, et al. 2011), and Tanzania (43.4) (O'Brien 2010). The highest incidence of the disease in our study was observed between age of 30-39 (36.5%) and the lowest was found with age of >60 years (10.6%). This finding was also in agreement with the previous study by Kantelhardt, et al. (2014a) among Ethiopian female breast cancer cases. On the contrary, a large cohort study in USA showed only 6.4% of the breast cancer cases were diagnosed at age younger than 40 years old (Gnerlich, et al. 2009). Generally, Sub-Saharan African countries reported younger age at presentation as compared to European and White American female breast cancer cases (Fregene and Newman 2005; Jedy-Agba, et al. 2016; Stark, et al., 2010).

The more possible reason for higher frequency of younger age at diagnosis in the current study might be due to the higher younger age population distribution in Ethiopia similar to other Sub-Saharan countries (Central Statistical Agency [Ethiopia], 2014; Jedy-Agba, et al. 2016). The higher incidence of younger age at diagnosis in this study as compared to Europeans/ White-Americans may also partly arise from the racial disparities and associated genetic variation between Africans and Europeans/ White Americans including BRCA1 and BRCA2

(Fackenthal, et al. 2005; Newmann 2014). Moreover, higher level of free estradiol and lower level of sex hormone binding globulin were reported in premenopausal Africa-American than white Americans (Pinherio, et al. 2005). However, the precise reason for the higher incidence rate of early age presentation in Sub-Saharan population than high-income countries is not conclusive yet.

With regard to the histologic subtypes, invasive ductal type was most common in this study (89.2%). Previous studies in female population also revealed invasive ductal carcinoma as the most common type of breast cancer which ranges from 70 to 79% (Kantelhardt, et al. 2014a; Kantelhardt, et al. 2014b; Shenkutie, et al. 2017). The slight increase in its frequency for the current study might be due to difference in classification scheme between the current and previous studies. Breast cancer studies from other African countries such as Uganda (87.6%) (Galukande, et al. 2014), Tanzania (88.6%) (Mwakigonja, et al. 2017), South Africa (80.0%) (McCormack, et al. 2013), Sudan (90% and 87.9%) and Eritrea (76.7%) (Galukande, et al. 2014; Sengal, et al. 2017) also showed that IDC was the most common type. This study, similar to previous studies in Ethiopian women and other African countries, revealed that there was higher frequency of IDC of breast cancer. The higher incidence of IDC in our study might be due to age distribution of the study participants and also genetic variation with women from other countries. Studies showed that higher incidence of IDC in African breast cancer patients, especially in younger age groups, have increased risk of developing primary invasive cancer in women with first diagnosed with DCIS than Europeans and Caucasians (Bowen, et al. 2008; Nassar, et al. 2009; Rambau, et al. 2011; Sanders, et al. 2005; Warnberg, et al., 2000). The occurrence of other histologic types observed in the present study was also in agreement with in other studies (Arpino, et al. 2004a; Winston, et al. 2000).

In the current study based on histopathological examination, pT3 was the most frequent type (32.5%) and majority of the tumor tissues (>60%) were pT3/pT4. Whereas, the previous study by Shenkute, et al. (2017) reported T2 as the most frequent tumor size (44.9%). Another study in Ethiopia by Kantelhardt, et al. (2014b) reported the mean size of 4.96 cm (near to pT3) for the breast tumor size. The difference in the frequency between the present and the previously reported results might be due to difference in the study participants and/or different classification system. Although we did not assess the stage of the disease due to lack of clinical data for presence or absence of metastasis, the higher incidence of large size tumors and lymph node positivity as well as the significant association between the two prognostic factors might reflect the delayed presentation/ diagnosis in the current study. Generally, late-stage presentation or diagnosis was common in Sub-Saharan Africa as compared to high-income countries (Benbakhta, et al. 2015; Grosclaude, et al. 2001; Pruitt, et al. 2015; Tetteh & Faulkner 2016). Hence, the higher frequency of large-sized tumors in the current study was probably due to the delayed presentation/ diagnosis since it is associated with larger tumor size (Caplan 2014; Jedy-Agba, et al. 2016; Odongo, et al. 2015). Delayed presentation or diagnosis could result from lack of awareness for early detection, socio-culctural and socio-economic factors and poor health facilities that are also major problems in African countries (Akuoko, et al. 2017; Brinton, et al. 2014; Tetteh & Faulkner 2016).

In the current study, half of the total tumors were found as poorly differentiated followed by moderately differentiated tumors (38%) according to the modified SBR grading system. This result was close to the finding by Jiagge, et al (2016). On the other hand, two other previous studies in Ethiopian population reported G2 as the most frequent (51.5% and 57.2%) followed by G3 (35.4% and 24.8%) (Kantelhardt, et al. 2014b; Shenkutie, et al. 2017). Studies from

Eritrea (Sengal, et al. 2017) and Kenya (Bird, et al. 2008) reported the prevalence of G3 tumors similar to our finding. On the other hand, a little higher frequency of G3 tumors than our current finding was also reported from Eritrea (Tesfamariam and Roy 2013), Sudan (Awadelkarim, et al. 2008), Uganda (Galukande, et al. 2014), Nigeria (Minoza, et al. 2016), and Tanzania (Abdulrahman and Rahman 2012). The proportion of poorly differenciated tumors showed variation in the previous studies among Finnish and Nigerian women (Ikpatt, et al. 2002) as well as among black and white British women (Bowen, et al. 2008). Generally, Africans and Africa-Americans were presented with increased rate of G3 breast cancer tumors as compared to White-Americans and Europeans (Batina, et al. 2013; Boder, et al. 2011; Ikpatt, et al. 2002; Morris 2008). Hence, the larger proportion of G3 tumors than G1/G2 in the current study might reflect aggressive nature of breast cancer in Ethiopian women. Such differences might be associated with differences in expressions of cell cycle regulatory proteins (Gukas, et al. 2005; Porter, et al. 2004). Poorly differenciated tumors were associated with increased risk of 10 year recurrence for low stage disease and risk of poor 5-year survival in invasive breast cancer (Rakha, et al. 2008).

In the present study, majority (74%) of the breast tumors were found as positive for lymph node involvement. This result was slightly lower than the previous study by Kantelhardt, et al. (2014b) but higher than Shenkutie, et al. (2017) who reported 80.8% and 52.3% of lymph node positivity in Ethiopian women respectively. The variation between the findings might be due to the difference in the type of study sample because both invasive and in situ breast tumors were included in the previous study. The current result was also nearly similar with the finding from Algeria (70%) (Souad, et al. 2018) but different lymph node positivity was reported from two studies among Sudanese women (53% in one and 90% in the other) (Awadelkarim, et al. 2008;

Sengal, et al. 2017). On the other hand, lower incidence of lymph node positive tumors was reported from Caucasian women (Furberg, et al. 2001). The higher incidence of lymph node positivity in the current study may mainly results from the delayed presentation or diagnosis which lead to increased spread of the tumor to the lymph nodes. Various studies also reported increased rate of lymph node metastasis either due to delayed diagnosis in Africa-Ameriacans and Black Eurpeans as compared to their Caucasians counterpart (Bowen, et al. 2008; Caplan 2014; Fregene and Newman 2005; Gajdos, et al. 2000; Kroman, et al. 2000; Zabicki, et al. 2006).

Expressions of the IHC markers

In the present study, majority of the breast tumors were positive for ER and PR expressions (73.8% and 60.2% respectively) in agreement with the findings by Jiagge, et al. (2016) who reported 71.4% of ER positive breast tumors in Ethiopian women. Previous studies also found 65% ER positive and 58% PR positive tumors in Ethiopian female breast cancer studies (Kantelhardt, et al. 2014a; Shenkutie, et al. 2017). Similar to our findings, East-African countries also reported higher hormonal receptor positivity from Eritrea (64.0 % and 49.1% of ER+ and 40.5% PR+) (Sengal, et al. 2017; Tesfamariam and Roy 2013) and Sudan (64% and 45% ER+, 67% and 38.2% PR+) (Awadelkarim, et al. 2008; Sengal, et al. 2017). Nearly similar frequency of ER positivity was also reported from Uganda and Egypt (Roy and Othieno 2011; Salhia, et al. 2011). Whereas, lower proportions of hormonal receptor expressions were reported from Nigeria, Ghana and Senegal (Gukas, et al. 2005; Huo, et al. 2009; Ikpatt, et al. 2003; Jiagge, et al. 2016; Omoniyi-Esan, et al. 2015). Generally, ER positivity was reported from 20%-70% in Sub-Saharan countries, whereas, 40%-80% in North Africans (Eng, et al. 2014). On the other hand, the prevalence of ER and PR positivity were mostly reported 65%-80% and

65%-75% respectively among white Americans and Europeans (Dai, et al. 2016; Joslyn and West 2000; Stead, et al. 2009).

Our study showed high hormone receptor expression similar to other East Africans and Caucasians but different from West Africans and Africa-Americans. A large cohort of age-adjusted breast cancer study from USA also revealed that East African-born immigrants were similar with White Americans, whereas, West-African born Americans were similar with Africa-Americans regarding ER status (Jemal and Fedewa 2012). Hence, this study also reflects the variation of hormonal receptor expression among African countries. The disparity in hormonal receptor expression between the current study and previous studies might partly due to ethnicity and also use of diverse pre-analytical and analytical techniques (Bhikoo, et al. 2011; Brown, et al. 2008; Eng, et al. 2014; Galukande, et al., 2013; Sofi, et al. 2012). Althogh further study is required for conclusion; the higher hormone receptor positivity in the current study might signify the benefit of hormonal therapy for Ethiopian breast cancer cases even though analysis of hormone receptor status as a routine procedure for diagnosis is not feasible in such resource-poor settings.

Due to breast cancer molecular heterogeneity, it is difficult to predict its clinical progression and selection of appropriate therapy only by using the standard prognostic and predictive markers including ER, PR and HER2 (Falchook, et al. 2013; Wahid, et al., 2017). This necessitates identification of additional potential biomarkers associated with tumor growth, angiogenesis and metastasis. The study of these biomarkers in particular group of people provides further understanding of the disease characteristics and helps for selection of appropriate treatment plans (Gilarranz 2012). Various studies indicated the important role of EGFR and VEGF-A in different types of cancers including breast in promoting tumor growth,

invasion and metastasis (Laskin and Sandler 2004; Massarweh, et al. 2008; Massarweh and Schiff 2006; Mohammed, et al. 2007).

In the current study, 21.7% of the tumor samples were EGFR positive and it is the first time study for assessment of EGFR status among breast cancer cases in Ethiopian population. Limited data is available regarding the prevalence of EGFR overexpression in breast carcinoma and its relationship with other clinico-pathological features especially in Sub-Saharan Africa. The prevalence of EGFR overexpression in the current study was somehow lower than the Tunisians (28.6%) (Kallel, et al. 2012) but nearly similar with European (20.6%) (Park, et al. 2007) invasive breast cancer cases. On the other hand, Rimawi, et al. (2010) reported slightly higher incidence in Black American (25.2%) but lower in White American (17.5%) invasive breast tissues than our current finding. Generally, overexpression of EGFR was reported with the range of 16% to 36% in breast cancer studies (Zeng, et al. 2011). The variation between this study and the previous findings in the proportions of EGFR overexpression might be due to the small sample size, type of specimens, sample processing and lack of standardized scoring system. In this study, EGFR positivity was considered only for specimens with strong membranous staining of >10% of the observed tumor cells and the rest were considered negative. In other studies, tumors with weak/ moderate membranous staining were also considered positive and showed EGFR amplification (Altaf, et al. 2014; Nakajima, et al. 2014; Shao, et al. 2011). Such challenges even may result in variable findings between different studies within the the same population as shown in Greece by Magkou et al (2008) and Koletsa et al (2010). Hence, standardized protocol is necessary to assess the EGFR status of Ethiopian breast cancer cases in future studies. EGFR over expression has been associated with increased cell growth, poor prognosis, resistance to chemotherapy and higher risk for relapse (Arpino, et

al. 2004b; Dowsett, et al. 2006; Grunwald and Hidalgo 2003; Koletsa, et al. 2010; Ritter and Arteaga 2003). Whereas, such kind of association was lacking in other studies (Ferrero, et al. 2001; Gori, et al. 2009; Rampaul, et al. 2004).

According to the present findings, 63.9%, 20.5% and 2.4% of the breast tumors showed low, moderate and high VEGF-A expression respectively. Although limited data were available from African countries, a study from Egypt reported 60% of VEGF-positivity among non-metastatic female breast carcinoma cases (Ragab, et al., 2016). Norwegian invasive breast cancer study reported 43.4% and 4.3% of moderate and high VEGF expression respectively (Dhakal, et al. 2012). Another study from Europe also showed 87.15% of VEGF positive invasive breast tumors similar to our finding (Cîmpean, et al. 2008).

Hence, as shown from the current and previous studies regarding VEGF-A status, variations in reported proportions VEGF-A expression in breast cancer were observed. One reason for the variation might be due to differences in methodologies including sample size, variability in tissue fixation, processing, type of specimens, scoring system due to lack of standards for cut-off values. The other possible reason would be genetic variations as a result of single nucleotide polymorphisms (SNPs). Several types of SNPs in VEGF-A were studied among different population that were associated with the risk of breast cancer (Jacobs, et al. 2006; Jin, et al. 2005; Kidd, et al. 2010; Schneider, et al. 2008). The genotypes of VEGF-A gene containing -1154GG and +405GG were associated with increased production of VEGF-A protein in breast cancer cases by Rani, et al. (2014). Among the VEGF family members, the expression of VEGF-A and its association with clinico-pathological features were studied more to be associated with poor prognosis, disease relapse and shorter overall survival in various breast

cancer studies (Berns, et al. 2003; Foekens, et al. 2001; Linderholm et al. 2009b; Manders, et al. 2003).

Interassociations of ER, PR, EGFR and VEGF-A

In the current study, ER expression was significantly correlated with expression of PR by the breast tumors (79% vs. 4.8%). Similar finding was reported in Ethiopian women (Kantelhardt, et al. 2014b) as well as other African breast cancer studies (McCormack et al., 2013; Minoza et al., 2016) and elsewhere (Ali, et al. 2014; Qiao et al., 2013; Ratnatunga, et al. 2007). Hence, the significant association between the two hormonal receptors in the current study was most probably due to activation of PR synthesis via ER-dependant mechanism (Grann, et al. 2005; Liu, et al. 2010).

Our study showed significantly inverse association between hormonal receptors and EGFR expression. EGFR positivity was more frequent in ER negative tumors as compared to ER positive ones (42.9% vs. 14.5%). Similarly, the frequency of EGFR over expression was higher in PR-negative tumors than PR-positive (33.3% vs 14.0%). Our findings were in agreement with the inverse relationship revealed in other studies (Arpino, et al. 2004b; Magkou, et al. 2008; Nieto, et al. 2007; Rampaul, et al. 2005; Rimawi, et al. 2010; Suo, et al. 2001; Tsutsuiet, al. 2002b; Witton, et al. 2003; Zheng, et al. 2015b) although some studies did not find significant association between EGFR and hormone recptor statuses (Gasparini, et al. 1992; Qiao, et al. 2013; Skobe, et al. 2001). The inverse correlation observed in the current study might be due to down regulation of hormone receptor expression by EGFR signaling (Arpino, et al. 2005; Dowsett, et al. 2006; Huang, et al. 2005; Konecny, et al. 2003; Rampaul, et al. 2005; Skasko, et al. 2005; Tsutsui, et al. 2002b). Althogh these receptors showed inverse association,

expression of each of these receptors play a role in the growth of breast tumors (Martin, et al. 2014; Quintela, et al. 2005). Moreover, the cross-talk between signaling pathways of EGFR and ER were revealed to be one of the mechanisms for resistance against endocrine therapies (Arpino, et al. 2008; Schiff, et al. 2004).

This study did not find significant association between VEGF-A positivity and hormone receptor status. Various studies also revealed no significant association between expression of VEGF and hormonal receptor status (Buteau-Lozano, et al. 2002; Fuckar, et al. 2006; Gunningham, et al. 2000; Linardou, et al. 2012; Linderholm, et al. 2000a; Ragab, et al. 2016; Srabovic, et al. 2013; Yavuz, et al. 2005). Other studies showed the inverse relationship between VEGF expression and the hormonal receptors (Foekens, et al. 2001; Fuckar, et al. 2006; Linderholm, et al. 2000b; Liu, et al. 2011) On the contrary, Adams, et al. (2000) reported the positive relationship ER and VEGF-A positivity. Although the current study did not find statistically significant association, the ER positive tumors were more frequent to be VEGF-A positive than ER negative tumors (25.8% vs 14.3%). Since most of the tumors were ER positive, the current finding might be associated with ER-induced VEGF-A synthesis in ER positive tumors (George, et al. 2012; Liang, et al. 2006; Stein, et al. 2009) or inhibition of VEGF-A synthesis in ER negative tumors (Wang, et al. 2018). Co-expression of hormone receptors and VEGF were shown to be associated with shorter recurrence-free and overall survival of the patient even after adjuvant endocrine treatment (Berns, et al. 2003; Linderholm, et al. 2009a; Liu, et al. 2011).

Associations of Age with Expression of the IHC Markers

Since the menopausal status of the study participants were lacking, the current study participants were divided into two age groups (younger age group, <50 years and older age group, >50 years) for comparative analysis with other characteristics of breast tumors. Although age groupings were variable, most of the breast cancer studies, cited in this thesis, were dichotomized more or less similar to the current study. In this study, no significant association was observed between hormone receptor status and the age groups (younger and older) although hormonal receptor positive tumors were more frequent among older age cases. Our finding also corroborated with Bird, et al. (2008) in Kenyan women. Previous study reported by Kantelhardt, et al. (2014b) revealed an association between younger age and ER negativity among Ethiopian breast cancer cases. Young age was associated with hormonal receptor negativity as shown in various studies (Alieldin, et al. 2014; Bauer, et al. 2007; Cluze, et al. 2009; Dobi, et al. 2011; Ibrahim, et al. 2014; Kataoka, et al. 2014). But our finding did not reach statistical significance that might be due to smaller sample size, type of fixative and fixation time (Engel and Moore 2011).

In the present study, the association of EGFR overexpression and age did not reach statistical significance although EGFR positivity was more frequent in patients younger than 50 years of age compared to older ones (24% vs. 16%). In another study, a large cohort study by Rimawi, et al. (2010) reported the direct association of EGFR positivity with younger age (<50 years) invasive breast cancer cases. EGFR overexpression was also associated with premenopausal breast cancer cases (Zheng, et al. 2015b). We did not find significant association of VEGF-A expression with age in the current study although its incidence was more frequent in older age groups. Similar findings were reported in other studies (Almumen, 2015; Ghasemi, et al. 2011;

Zhu, et al. 2016). However, VEGF-A expression was associated with older age in node-negative breast cancer cases in another study by Sa-nguanraksa and O-charoenrat (2012) and opposite result was reported by Fuckar, et al. (2006).

Association between Age and Histopathological Features

Younger age at diagnosis was significantly associated with large tumor size in our study but no association was seen between age and tumor grade in agreement with Ali, et al. (2014) found in Egyptian women. Higher lymph node involvement (pN2/N3) was also more frequent in younger age group as compared to older age groups in our study although not statistically significant (40% vs. 25%). Various studies reported the commonness of bigger tumor size, higher tumor grade and increase in lymph node involvement among younger age female breast cancer cases (Andres, et al 2009 ; Bardou, et al. 2003; Joensuu, et al. 2003; Kroman, et al. 2000; Rapiti, et al. 2005; Shannon and Smith 2003; Yao, et al. 2009).

The higher frequency of larger tumor size and axillary lymph node involvement at younger age in the present study as compared to older age breast cancer cases might be partly due to the increased frequency of hormone receptor-negative tumors at younger age than older age groups. As compared to both ER-positive and PR-positive tumors, ER-positive/PR-negative and ER-negative/PR-negative tumors were more characterized by larger in size and lymph node positive (Dai, et al., 2016; Neven, et al., 2006; Thakkar and Mehta 2011). The increased incidence of larger tumor size in younger than older age cases in the current study might also be due to the higher level of free estrogen among younger age groups. Free estrogen level was shown to be higher in premenopausal than postmenopausal women due to lower level of estrogen-binding globulin. In turn, estrogen promotes proliferation of breast cancer cells (Setiawan, et al. 2006; Tian, et al. 2018). The small sample size in the current study might be the reason for lack of

significant association between age at diagnosis and axillary lymph node involvement. Lack of association between age and tumor grade in the current study might also show the aggressive nature of the disease even at older age.

Association of IHC Markers Expression with Histopathologic Features

We did not find any significant association between hormonal receptor status and the size of breast tumor tissue in agreement with the findings from previous studies (Ayadi et al., 2008; Bamberger, et al. 2000; Fatima, et al. 2005; Kilinç and Yaldiz 2004; Mudduwa. 2009; Rosenberg, et al. 2006). The present study also found no association between expressions of hormonal receptors and lymph node metastasis. Our findings were in agreement with previous studies (Azizun-Nisa, et al. 2008; Chua, et al. 2001; Pourzand, et al. 2011). But significant positive and negative associations were reported by Zhou, et al (2014) and Ali, et al (2014) respectively. Our study did not show significant association between hormone receptor status and histologic grade in agreement with the previous findings (Aryandono and Soeripto 2006; Kamil, et al. 2010). However, hormone receptor postitive tumors were commonly associated with well/ moderately differenciated tumors (Nadji, et al. 2005; Rosa, et al. 2008; Sengal, et al. 2017). The higher proportion of ER positive G3 tumors than ER negative counterparts in the current study might be related with the heterogeneity of ER positive tumors and thus further studies are needed regarding ER-regulated genes (Loi, et al. 2007; Oh, et al. 2006; Thakkar and Mehta 2011).

In the present study no significant association was found between EGFR over expression and tumor size although the incidence of overexpression was greater in large size as compare to small size tumors. Similar finding was reported in previous studies (Giltnane, et al. 2007;

Magkou, et al. 2008; Quao, et al. 2013). However, EGFR overexpression was shown to be significantly associated with tumors >2cm in size among USA females in a large cohort study (Rimawi, et al. 2010). The difference with the current finding might be due to small sample size, differences in tissue processing or analytical method and cut-off values. The present study did not show significant association between EGFR overexpression and lymph node metastasis. Non-significant association was also reported in previous studies (Fox, et al. 1994; Magkou, et al. 2008). However, significantly inverse association was reported by Dihge, et al. (2008) among UK females.

Regarding the association between EGFR status and histologic grade, this study did not show significant association between them. But, EGFR positivity was more frequent in poorly differentiated than low/ intermediate grade tumors. Although they found significant association with nuclear grade, numerous studies reported non-significant association of EGFR positivity with histologic G3 tumors similar to the current finding (Arpino, et al. 2004b; Magkou, et al. 2008; Rampaul, et al. 2005). Whereas, other studies reported significant association between EGFR overexpression and high grade tumor in invasive type of breast carcinoma (Aboushousha, et al. 2018; Giuliania, et al. 2007; Yao, et al. 2017). EGFR positive breast cancer cases were shown to have shorter survival associated with poor clinical outcome (Park, et al. 2007; Suo, et al. 2002).

In this study, no significant association was observed between VEGF-A status and tumor size although a slightly increased frequency of VEGF-A expression in tumors > 5cm in size. This finding was in agreement with the findings of previous studies (Ali, et al. 2011; Almumen 2015; Cimpean, et al., 2008; Ludovini, et al. 2003; Meunier-Carpentier, et al., 2005; Nieto, et al. 2007; Ryden, et al. 2005; Srabovic, et al. 2013; Valkovic, et al. 2002). Whereas, Mohammed, et al

(2007) from UK reported significant correlation between VEGF-A expression and tumor size > 1.5 cm. Other studies also revealed significant association between breast tumor size and VEGF expression (Ali, et al. 2011; Linderholm, et al. 2008). On the contrary, VEGF-A expression and tumor size showed inverse relationship in other studies (Comsa, et al. 2012; Halimi, et al. 2012). The difference in the current finding from some studies might be due to the difference in the grouping of tumor size as small or large. Although VEGF-A was well known an angiogenic factor and important for tumor growth, the lack of association between tumor size and VEGF-A expression in this study might partly due to increased secretions of other angiogenic factors such as fibroblast growth factor which contributed to the larger sized tumors in the current study apart from VEGF-A (Hanahan and Weinberg 2011). The other reason for lack of association between VEGF A expression and tumor size might be due to the minimal effect of autocrine signaling of the ligand on the tumor cells since VEGF-A primarily binds to endothelial cells to promote angiogenesis (Ellis 2004).

In the present study, no significant difference in VEGF-A positivity was observed among histological grades of the breast tumors. Nearly 20% of both lower and higher grades of the tumors were found to be VEGF-A positive in this study. Similar to the current finding, several studies reported no significant association between histologic grade of breast tumors and VEGF status (Chen, et al. 2015; Ludovini, et al. 2003; MacConmara, et al. 2002; Meunier-Carpentier et al. 2005; Nieto, et al. 2007; Ryden, et al. 2005). In other breast cancer studies, inconsistent results were reported regarding the association between VEGF-A status and tumor grade. Some studies reported positive association between high VEGF expression and poorly differentiated tumors (Konecny, et al. 2004; Mohammed, et al. 2007; Shankar, et al. 2006) while others found

inverse relationship between them (Adams, et al., 2000; Al-Bassam, et al. 2014; Ghasemi, et al. 2011).

In this study, no significant association was observed between VEGF-A expression and lymph node status. Similar results were reported in other studies (Ali, et al., 2011; Almumen 2015; Cimpean, et al. 2008; Comsa, et al. 2012; Dhakal, et al. 2012; Qiao, et al. 2013). Some studies found positive associations (Al-Harris, et al. 2008; Mohammed, et al. 2007). Although no significant correlation between VEGF-A expression and tumor size or lymph node status, the VEGF-A positivity could be associated with metastasis to other organs independent of tumor size or nodal metastasis (Dvorak 2002). VEGF-A is secreted by the tumor cells but the receptors for the ligand were found to be predominantly expressed by the endothelial cells in breast cancer (Ellis 2004). Hence, the lack of association between VEGF-A expression and the morphological features (tumor size, nodal status and grade) in the current study might be due to lack of direct effect of the VEGF-A on the tumor as a result of minimal expression of VEGFR2, the major VEGF-A receptor, by the tumor cells. Although the expression of VEGFRs by breast tumor cells was controversial, studies found low expression VEGFR2 by breast tumor cells (Lee, et al. 2007; Miettinen, et al. 2012; Nasir, et al. 2017). However, VEGF-A expressed by the breast tumor was known to mediate the expression of other angiogenic molecules that play important role in tumor angiogenesis (Ryan, et al. 2000).

Interrelationships between Histopathological Characteristics

The current study revealed significant association between tumor size and lymph node metastasis of the breast tumors. The incidence of higher lymph node involvement in large size tumors was much higher than small size tumors (48.9% vs 10.3%). Several breast cancer studies

revealed the correlation between larger tumors with lymph node metastasis (Akasbi, et al. 2011; Dent, et al. 2007; Patani, et al. 2007; Siddiqui, et al. 2002; Xie, et al. 2012; Yoshihara, et al. 2013). But others reported no significant association between tumor size and lymph node involvement (Cheang, et al. 2008; Derkaoui, et al. 2016). Tumor size of the breast cancer has been established as a prognostic factor and as it enlarges, increases the occurrence of axillary lymph node involvement (Lee, et al. 2010; Silverstein, et al. 2001).

We did not find any significant association between tumor grade and axillary lymph status. Several studies also revealed the positive correlation between tumor grade and axillary lymph node involvement (Khan, et al. 2001; Patani, et al. 2007; Siddiqui, et al. 2002; Xie, et al. 2012; Yoshihara, et al. 2013). The absence of association between the grade and nodal involvement might be due to sample size and/ or difference in analytical method in addition to biological differences. Histologic grade and lymph node status have been considered as powerful prognostic factors and related with survival (Chakraborty, et al. 2016; Desmedt, et al. 2008; Esteva and Hortobagyi 2004). In this study, high grade tumors were more likely associated with larger size tumors but the association did not reach statistical significance ($p=0.07$). Although the relation between tumor size and histologic grade is not certain, it tells about the tumor character and the evidence is important for selection of therapeutic options (Schwartz, et al. 2014).

6. CONCLUSIONS

- Assessment of the histopathologic features in the current study samples revealed the predominance of younger age presentation, larger tumor size, higher axillary lymph node positivity and poor differentiation of tumors which are all indicating aggressive nature of the tumor samples regardless of the age categories.

- Majority of the breast tumors were positive for expressions of ER and PR which seems somehow similar to white Europeans and Americans.

- Although limited data has been found, EGFR positivity currently observed in Ethiopian women was similar with the reported value from the database in Africa-Americans but higher than Whites.

- Majority of the breast tumors in the current study expressed VEGF-A although the proportion of tumors with intermediate/ high level expression was limited.

- Age at diagnosis in this study was not significantly associated with ER, PR, EGFR and VEGF statuses although hormone receptor positivity was more frequent at older age. Age was inversely correlated with tumor size but it did not show significant correlation with axillary lymph node involvement and tumor grade.

- In the current study, we did not find any significant association of hormone receptors expression with tumor size, histologic grade and axillary lymph node involvement. Similarly, expressions of both EGFR and VEGF-A were not correlated with the morphological features. EGFR overexpression was inversely correlated with hormone

receptors expression whereas VEGF-A showed no significant association with them. Hence, EGFR might be a good predictor in adjuvant therapy for hormone receptor negative breast cancer cases. But, further study is necessary to clarify this finding in Ethiopian women.

- In the current study, axillary lymph node status was found to be positively correlated with tumor size but not with histologic grade. Hence, nodal metastasis of breast cancer in Ethiopian women seems to be associated with the increase in tumor size.

7. LIMITATIONS OF THE STUDY

The major limitations of our study are inadequacy of sample size and lack of clinical data. Future studies should be done on large number of samples and clinical information should be incorporated for further understanding of the disease behavior. This study also lacks assessment of other important biomarkers, such as HER2, to categorize breast tissues based on molecular subtypes because of other constraints including budget.

The number of biomarkers evaluated in this study was limited. These markers may not be sufficient to understand the molecular characteristics of the disease. VEGF-A receptor status and intratumoral Microvessel density (MVD) was not assessed in our study. Hence, the effect of VEGF-A expression could not be determined with out these parameters.

8. RECOMMENDATIONS

- Prospective studies with adequate clinical and histologic information of the breast tumor as well as analysis of additional molecular markers should be done to understand the disease behavior in Ethiopian women.

- Empasis should be given how to improve community awareness self examination and presentation to health centers for breast cancer and also improving health facilities for early diagnosis.

- At least ER and PR status should be determined as part of routine diagnostic procedure if possible to determine the status and decide the appropriate treatment option. Since majority of the tissues revealed hormone receptor positivity, endocrine therapes might benefit majority of the breast cancer in Ethiopian women although receptor statuses might not be routinely assessed due to lack of facilities.

- The routine handling of sample and pathologic procedures should be optimized to the appropriate standard for better evaluation of the immunohistochamical surrogates.

- Improved methodologies should be applied to assess the status of VEGF-A and EGFR biomarkers to get more reliable results.

- Assessment of additional potential biomarkers should be done to understand the tumor biology more in detail to confirm which molecular factors play a role in breast carcinogenesis in Ethiopian women.

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Annex IA. Ethical Approval Letter from Institutional Review Board, CHS, AAU

	Addis Ababa University College of Health Science Institutional Review Board **Title:** **3.2. Use of Study Assessment Form**	**SOP# AAUMF 008** **Version 2.0** **Effective date: 1 Feb. 2009** **Page 13 of 13**

ANNEX 3
Form AAUMF 03-008

IRB's Decision

Meeting No: 059/14 Date (D/M/Y): April, 2014
Protocol number: 010/14/Bioch Assigned No.....

Protocol Title: Characterization of breast cancer subtypes by surrogate biomarkers and VEGF gene copy number variation among female breast cancer cases admitted to TASH	
Principal Investigators:	Sisay Addisu
Institute:	School of Medicine-College of Health Sciences, AAU
Elements Reviewed (AAUMF 01-008) :	☑ Attached ☐ Not attached
Review of Revised Application ☐ Yes ☐ No	Date of Previous review:
Decision of the meeting:	☑ **Approved** ☐ Approved with Recommendation ☐ Resubmission ☐ Disapproved

I. Elements approved-
1. Protocol Version No. .....
2. Protocol Version Date.....
3. Informed consent Version No. .....
4. Informed Consent Version Date .....

II. Obligations of the PI-
1. Should comply with the standard international & national scientific and ethical guidelines
2. All amendments and changes made in protocol and consent form needs IRB approval
3. The PI should report SAE within 10 days of the event
4. End of the study, including manuscripts and thesis works should be reported to the IRB

III. **TO NERC** ☑
Institution Review Board (IRB) Approval: Period from **15 May 2014 to 14 May 2015**
Follow up report expected in
3 Months ___ 6 months ____ 9 months ✓ one year____

Chairperson, IRB
Dr. Yimtubezenash W/Amanuel
Signature _____
Date: _____

Associate Dean Director of Research and Technology Transfer
Signature _____
Date _____

Annex IB. Ethical Approval Letter from Institutional Review Board, CHS, AAU

ADDIS ABABA UNIVERSITY COLLEGE OF HEALTH SCIENCES (IRB)
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Institutional Review Board

ANNEX 3
Form AAUMF 03-008

IRB's Decision

Meeting No: 007/2015 Date: August 20, 2015
Protocol number: 010/14/Bioch. Assigned No.....

Protocol Title: Characterization of Breast Cancer Sub-types by Surrogate Bio-markers and VEGF gene copy number variation among female breast cancer cases admitted to TASH		
Principal Investigators:	Sisay Addisu	
Institute:	CHS-AAU	
Elements Reviewed (AAUMF 01-008)	☑ Attached	☐ Not attached
Review of Revised Application ☐ Yes ☐ No	Date of Previous review:	
Decision of the meeting:	☑ **Approved** ☐ Approved with Recommendation ☐ Resubmission ☐ Disapproved	

I. Elements approved-
1. Protocol Version No. ...3.....
2. Protocol Version Date.....
3. Informed consent Version No. ...3.....
4. Informed Consent Version Date .....

II. Obligations of the PI-
1. Should comply with the standard international & national scientific and ethical guidelines
2. All amendments and changes made in protocol and consent form needs IRB approval
3. The PI should report SAE within 10 days of the event
4. End of the study, including manuscripts and thesis works should be reported to the IRB

Institution Review Board (IRB) Approval: Period extended from 20/08/2015 to 20/08/2016

Follow up report expected in
3 Months __ 6 months ___ 9 months__√_____one year____

V/ Chairperson, IRB
Dr. Adamu Addissie
Signature
Date: 20/08/15

ADDIS ABABA UNIVERSITY Institutional Review Board Office (IRB) Faculty of Medicine

Annex II. Ethical Approval Letter from Ministry of Science and Technology, Ethiopia

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የሳይንስና ቴክኖሎጂ ሚኒስቴር
The Federal Democratic Republic of Ethiopia
Ministry of Science and Technology

To: Addis Ababa University, Collage School of Medicine

Addis Ababa

ቁጥር 310/023/2015
Ref. No.

ቀን June 15, 2015
Date

Re: Characterization of Breast Cancer Subtypes by Surrogate Biomarkers and VEGF Gene Copy Number Variation among Female Breast Cancer Cases admitted to Tikur Anbesa Specialized Hospital

Dear Sir/Madam//Mr./Mrs./Dr,

The National Research Ethics Review Committee (NRERC) has reviewed the aforementioned project protocol in an expedited manner. We are writing to advise you that NRERC has granted

Full Approval

To the above named project, for a period of **one year (June 15, 2015- June 14, 2016).** All your most recently submitted documents have been approved for use in this study. The study should comply with the standard international and national scientific and ethical guidelines. Any change to the approved protocol or consent material must be reviewed and approved through the amendment process prior to its implementation. In addition, any adverse or unanticipated events should be reported within 24-48 hours to the NRERC. Please ensure that you submit biannual progress report once in six months and annual renewal application 30 days prior to the expiry date.

We, therefore, request you as PI and your esteemed organization to ensure the commencement and conduct of the study accordingly and wish for the successful completion of the project.

With regards,

Yohannes Sitotaw
Secretary of NRERC

CC. Mr. Sisay Addisu (PI)
_ Chairperson, NRERC

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You may Contact

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nnex III. Material Transfer Agreement

Ministry of Science and Technology, Federal Democratic Republic of Ethiopia
National Research Ethics Review Committee
Address: Tel. +251011-4-674353 P.O. Box 2490 Fax +251-011-4-660241
E-mail:nrerc2015@gmail.com Addis Ababa –Ethiopia

Material Transfer Agreement

This Material Transfer Agreement (MTA) has been prepared for use by **Department of Biochemistry, Addis Ababa University and University of Michigan** in all transfer of research material (samples, derivatives, and specimens) related to the protocol: **"Characterization of Breast Cancer Subtypes by Surrogate Biomarkers and VEGF Gene Copy Number Variation among Female Breast Cancer Cases"**

Provider: Department of Biochemistry, School of Medicine, Addis Ababa University
Recipient: Sofia Sofia D. Merajver (Professor) University of Michigan, USA

1. Provider agrees to transfer to recipient's designated (human biological sample) the following research materials (specimen) **Human Breast Tissue.**
   The research material will only be used for research purposes as described in the protocol by recipient's investigator in designated laboratory for the research project described below, under suitable containment conditions. This research material will not be used for commercial purposes such as screening, production or sale for which a commercialization license may be required. Recipient agrees to comply with all National and International guidelines rules and regulations applicable to the Research Project and the handling of the Research Material.
   a) Are the Research materials of human origin?
      Yes [X] No [ ]
   b) If yes, will they be collected according to the details in the protocol and in adherence to National Health Research Ethics Review Committee (NERC) and Addis Ababa University, College of Health Science Ethics Review Committee recommendations and their approval?
      Yes [X] No [ ]

1

2. This research material and its derivatives will be used by recipient's investigator solely in connection with the following research project ("Research Project") described with specificity as follows "Characterization of Breast Cancer Subtypes by Surrogate Biomarkers and VEGF Gene Copy Number Variation among Female Breast Cancer Cases."
3. In all presentations or written publications concerning the research projects, recipient will seek agreement of provider and acknowledge provider's contribution of this research material unless requested otherwise.
4. This research material represents a significant contribution on the part of provider and is considered proprietary to provider. Recipient therefore agrees to retain control over this research Material and further agrees not to transfer the research Material to other people not under her/his direct supervision without advance written approval of provider. The research material will be disposed of as agreed upon per protocol at the end of completion of the project on 2018.
5. The provider does not take any responsibility for loss, damage, wastage or spoilage of the research material during or after shipment to the address provided by the Recipient under conditions agreed to in the protocol on shipment of the samples. This Research Material is provided as a service to research community. IT IS BEING SUPPLIED TO RECIPIENT WITH NO WARRANTIES, EXPRESS OR IMPLIED, INCLUDING ANY WARRANTY OF MERCHANTABILITY OR FITNESS FOR A PARTICULAR PURPOSE. Provider makes no representations that the use of the research material will not infringe any patent or proprietary right or third parties.
6. The recipient shall notify the provider in writing of any intention, improvement, modification discovery or development to the material or the information made by Recipient or parities, collaborating with Recipient, herein after referred to an "invention". Nothing in this agreement shall, however, be construed as conveying to the provider any rights under any patents or other intellectual property to such invention, other than as explicitly provided herein. At its option the provider shall be entitled to receive sample of any materials derived from the Materials for its own research and evaluation purposes only.
7. The under-signed provider and Recipient expressly certify any affirm that the contents of any statements made herein are truthful and accurate.
8. Any additional terms (use an attached page if necessary):
9. The provider maintains, ownership right of the research material and its derivatives unless stated otherwise.

The provider will retain a copy (aliquot) of every sample sent abroad as much as possible for local research needs.

Material Transfer Agreement

Signature page

For Recipient:

Recipient's Investigator

Sofia D. Merajver, MD, PhD

Professor of Internal Medicine & Epidemiology

Scientific Director, Breast Oncology

Director, Breast & Ovarian Cancer Risk Evaluation Program

Phone: 734-936-6884

Signature

Date 8/17/2015

Mailing Address

1500 E. Medical Center Dr. 7217CC

Ann Arbor MI 48109

Tel: 734 936-6884

Fax: 734 615-2719

Duly Authorized

Signature/Stamp

Date

Mailing Address for Notices:

Tel:

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For Provider:

Provider's Investigator

Signature

Date

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Fax:

Duly Authorized

Signature

Date

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ADDIS ABABA UNIVERSITY Dept. of Biochemistry Medical Faculty A.A.